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THE MECHANICS OF THE SEMICIRCULAR CANAL

By A. A. J. van EGMOND, J. J. GROEN and L. B. W. JONGKEES

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When Steinhausen (1931) discovered that the cupula terminalis in the semicircular canal reaches the top of the ampulla, fitting hermetically in it, and when he moreover observed that a deviation of the cupula, caused by an endolymph flow, gradually decreases because of the directional force of the cupula, the theory of the semicircular canal underwent a significant change. Up till then there was held to be a narrow canal in which an endolymph-flow would soon be stopped by frictional damping. After a clinical turning test the endolymph would flow for about 0·5 sec. only, and then every part of the canal would be at rest. The prolonged after-sensations of 30 sec. or more could not be explained by the mechanical properties of the canal. They then had to be ascribed to a 'central origin', the only possible means of surmounting this difficulty (Mach, 1875; Gaede, 1922).

Steinhausen gave the cupula its proper prominence. The endolymph after a sudden arrest of the turning chair will indeed flow on for about 0·5 sec., but the cupula is forced to bend and after having attained its maximal deviation, without further external forces acting, it will try to recover its former position of zero deviation. This process may take from 1 to 60 sec., depending on the rate of the angular velocity just before the sudden arrest. The prolonged after-sensations appear to have a very simple mechanical origin. As Steinhausen pointed out, the cupula-endolymph system has to be considered as a heavily damped torsion pendulum. It thus has a differential equation with solutions available in every handbook on mechanics. This statement, important as it may be, brings us no further if the constants of the equation for this case cannot be determined, but if they are known this equation ought to predict any conduct of the cupula found in practice.

Steinhausen did not establish the values of the constants for his test animal, the pike. So he was unable to prove the hypothesis of the torsion pendulum with its consequences. We have determined these values for the human subject by means of experiments based on mechanical principles.

METHOD

A much clearer idea of the function of the semicircular canals can be obtained, using small stimuli, than is possible with the usual Bárány test.

The subject is placed in a turning chair, with the head inclined forward about 30°. The movement, either clockwise or anticlockwise, starts smoothly with a subliminal acceleration, which is maintained until the angular velocity needed is attained. It is halted quite suddenly (braking time 1–3 sec., depending on the angular velocity). The test subject has a sensation of turning round. This sensation expires after a certain period. The duration of the sensation is expressed as a function of the angular velocity. This relation is plotted in a diagram, which is called the cupulogram.

The test is performed with the test subject in darkness, lest he obtain his sensations from the surroundings. The determination of the duration of the nystagmus is performed with Frenzel's spectacles.

It is important that the test subject has no sensations of turning up to the time of braking.

The time, necessary to obtain the required velocity, depends on this velocity, but as a rule does not exceed 3 min. The angular velocities used lie between 1 and 60°/sec. The non-physiological velocities of Bárány of 180°/sec. and more were not used.

Comparison of the graphs of sensation and nystagmus duration gives a far better indication of any abnormality of function than the earlier methods.

For the vertical canals the same procedure can be followed, with the head in the appropriate position.

RESULTS

The differential equation

The differential equation of a torsion pendulum where there are no external forces is

$$\Theta\ddot{\xi}+\Pi\dot{\xi}+\Delta\xi=0 \quad \text{or} \quad \ddot{\xi}+\frac{\Pi}{\Theta}\dot{\xi}+\frac{\Delta}{\Theta}\xi=0. \tag{i}$$

The constants to be determined are Π/Θ and Δ/Θ:

Θ = moment of inertia of the endolymph.
Π = moment of friction at unit angular velocity.
Δ = directional momentum at unit angle caused by the cupula.
ξ = the angular deviation of the endolymph in relation to the skull.
$\dot{\xi}$ = the angular velocity of the endolymph in relation to the skull.
$\ddot{\xi}$ = the angular acceleration of the endolymph in relation to the skull.

(All these at the centre of the canal.)

The approximate solution of equation (i) is

$$\xi=\gamma\frac{\Theta}{\Pi}[e^{-\Delta t/\Pi}-e^{-\Pi t/\Theta}], \tag{ii}$$

with the limiting conditions: $t=0$, $\xi=0$ and $\dot{\xi}=\gamma$.

The angular velocity just before the sudden arrest of the turning chair is γ. It will be called 'the impulse', though it has not all the properties of a mechanical impulse.

Equation (ii) concerns the clinical turning test. After a period of turning with a constant velocity γ the subject is stopped: the endolymph will thus have the initial velocity γ, whereas the cupula had no deviation and was in perfect

equilibrium. The cupula is pushed forward and attains its maximal deviation at $t_{\max.}$:

$$t_{\max.} = \frac{\Theta}{\Pi} \log \frac{\Pi^2}{\Theta\Delta} \text{ (approx.)}. \tag{iii}$$

It then has a deviation $\xi_{\max.}$, where

$$\xi_{\max.} = \gamma \frac{\Theta}{\Pi} \text{ (approx.)}. \tag{iv}$$

At this moment the second term in equation (ii) $e^{-\Pi t/\Theta}$ is almost zero and may be neglected. The cupula then tends to regain equilibrium (zero deviation). This prolonged period is determined by the first part of equation (ii) $e^{-\Delta t/\Pi}$.

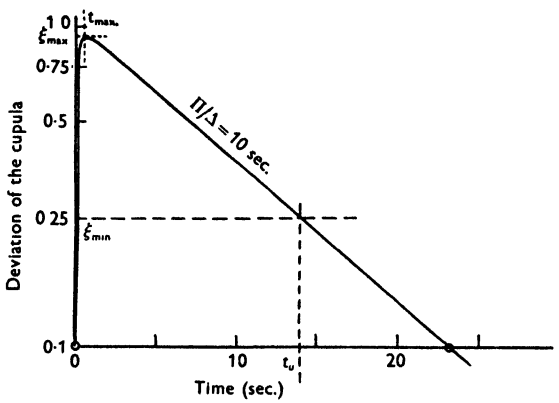

Fig. 1. Deviation ξ of the cupula produced by sudden arrest of an angular velocity γ°/sec. Maximum deviation at $t = 0{\cdot}5$ sec.; minimum deviation passed at $t = 14$ sec., giving rise to sensation. Ordinate logarithmic. Slope of curve gives Π/Δ.

The subject has a sensation of declining angular velocity with a direction opposite to the original rotation of the chair. This is quite plausible from a mechanical point of view. The sensation expires after t_u sec.

$$t_u = \frac{\Pi}{\Delta} \log \left(\frac{\Theta\gamma}{\Pi\xi_{\min.}}\right) \text{ (approx.)}, \tag{v}$$

where $\xi_{\min.}$ denotes the smallest deviation of the cupula giving rise to a just perceptible sensation of rotation. In Fig. 1 t_u is shown where the curve intersects with the $\xi_{\min.}$ line. This equation gives us a means of determining a ratio between two unknown constants of the differential equation. The value of t_u has to be determined as a function of γ and the slope of the curve gives Π/Δ, if the impulse is plotted logarithmically. This is done in Fig. 2. It appears that

$$\Pi/\Delta = 10 \text{ sec.}$$

The expectation that the curve is a straight line is confirmed. The smallest perceptible impulse $\gamma_{\min.}$ is about 1·5°/sec. This figure was obtained from a normal sensitive subject. All the sensitive subjects give values very close to

those of Fig. 2. The average values for all the normal subjects tested, the insensitive ones included, are

$$\Pi/\Delta = 8 \text{ sec.} \quad \text{and} \quad \gamma_{\text{min.}} = 2{\cdot}5^\circ/\text{sec.}$$

From equation (v) it follows that

$$\gamma_{\text{min.}} = \xi_{\text{min.}} \Pi/\Theta \text{ (approx.).} \tag{vi}$$

This agrees with $\xi_{\text{max.}}$ as expected.

Unfortunately, equation (iii) has no practical value for human subjects, as $t_{\text{max.}}$ has to be of the order of 0·5 sec. and this is too short a time to be estimated with satisfactory accuracy. Otherwise, with Π/Δ known, the differential equation would be determined.

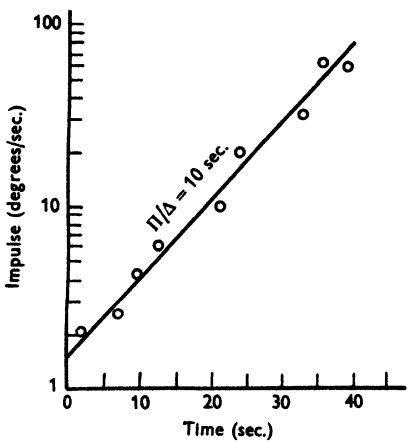

Fig. 2. Duration of sensation as a function of the impulse. Normal sensitive subject. Slope gives $\Pi/\Delta = 10$ sec. Minimal impulse ($\gamma_{\text{min.}}$) $= 1{\cdot}5^\circ$/sec.

Resonance vibrations on the torsion swing

A second experiment, independent of the former one, is needed to establish the value of Π/Θ. Resonance vibrations on a torsion swing enable us to impose upon the subject harmonic vibrations about a vertical axis. In this manner the horizontal canals are studied. The sensation of the subject is also a harmonic vibration, but there is a phase difference between sensation and swing. Only when the frequency of the swing is equal to the natural frequency of the canal is the phase difference zero. The natural frequency is that frequency which the cupula-endolymph system would have without damping, and is a theoretical value which cannot be demonstrated directly. The system does not vibrate when undisturbed. When a pendulum, damped or not, is forced to vibrate by means of a harmonic external momentum, it has a phase difference of 90° (lagging) when it is in resonance. When the external frequency is higher the phase difference becomes more than 90°, and in case of a lower frequency it

becomes less than 90°. When the swing reaches the turning point, the cupula, in case of resonance, is at zero deviation and the subject has the sensation of rest. The swing, coming from its turning point, will pass its zero deviation at its maximal velocity. The cupula at that moment has reached its turning point (and so its maximal deviation) and the subject has a sensation of maximal velocity. Thus sensation and vibration always coincide in case of resonance. But when the swing frequency is too high, the sensation of rest at the turning point will lag behind; when the swing frequency is too low the sensation will go ahead.

The theory in this case demands

$$\ddot{\xi}+\frac{\Pi}{\Theta}\dot{\xi}+\frac{\Delta}{\Theta}\xi=\alpha\sin\omega t, \tag{vii}$$

where α denotes the maximal angular acceleration and ω the frequency of the swing. α is connected to the amplitude A, and ω to the period T of the swing in the following way:

$$\alpha=\omega^2 A, \tag{viii}$$

$$T=\frac{2\Pi}{\omega}. \tag{ix}$$

The approximate solution of equation (vii) is

$$\xi=\frac{\alpha\Theta}{\omega\Pi}\sin(\omega t+\phi)+\ldots \text{ terms (negligible).} \tag{x}$$

The angle ϕ is determined by

$$\tan\phi=\frac{\omega}{\omega_0^2-\omega^2}\frac{\Pi}{\Theta}, \tag{xi}$$

where ω_0 is the natural frequency of the canal, and

$$\omega_0^2=\frac{\Delta}{\Theta}.$$

If it is possible to determine ω_0, Δ/Θ is calculable. When $\omega>\omega_0$, $\tan\phi<0$; thus $\phi>90°$ and the subject lags behind the swing. When $\omega<\omega_0$, $\tan\phi>0$, $\phi<90°$: the subject goes ahead of the vibration of the swing. The resonance frequency ought to lie between these two cases. Coincidence of sensation and swing itself does not suffice as an experiment, for it could be ascribed to a faulty observation. The phase differences to be expected are measurable as very short time differences of the order of 0·3 sec. at most. This is only possible with sensitive subjects. As an average value we found: $\omega_0=1{\cdot}0$ sec.$^{-1}$. Thus $\Delta/\Theta=1{\cdot}0$ sec.$^{-2}$. The differential equation (i) becomes

$$\ddot{\xi}+10\dot{\xi}+\xi=0. \tag{xii}$$

The probable error in Π/Θ is about 25% and in Δ/Θ about 20% (Egmond, Groen & Jongkees, 1943, 1948).

The measurements mentioned above may be checked as follow:

Test 1

In case of a circular movement with constant acceleration α rad./sec.^{2}, the behaviour of the cupula-endolymph system ought to be as follows:

$$\ddot{\xi}+\frac{\Pi}{\Theta}\dot{\xi}+\frac{\Delta}{\Theta}\xi=\alpha. \tag{xiii}$$

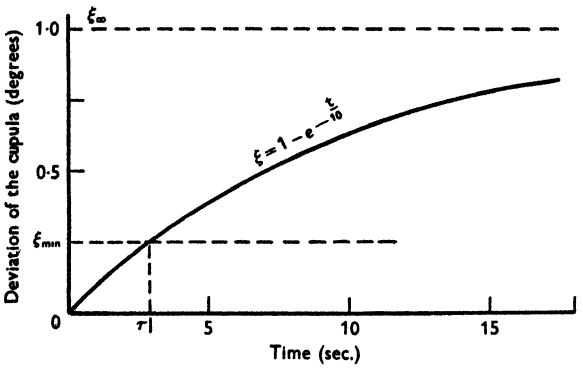

Fig. 3. Deviation ξ of the cupula under influence of a constant angular acceleration (1°/sec.)^2. The latent period τ seconds lies between start and the moment when the cupula passes $\xi_{min.}$. After infinite time (about 30 sec. in practice) the cupula attains equilibrium.

It has an approximate solution

$$\xi=\alpha\frac{\Theta}{\Delta}(1-e^{-\Delta t/\Pi}), \tag{xiv}$$

with the limiting conditions: $t=0$, $\xi=0$, $\dot{\xi}=0$, $\ddot{\xi}=\alpha$. The movement starts from rest; gradually the velocity γ will increase. After infinite time

$$\xi_\infty=\alpha\frac{\Theta}{\Delta}. \tag{xv}$$

When in the case of the human subject an angular acceleration of 1°/sec.^{2} is used, the deviation of the cupula with time may be estimated (Fig. 3). The formula is

$$\xi=1-e^{-t/10} \text{ degrees.}$$

At first the subject feels at rest until the cupula passes the $\xi_{min.}$ value; then the first sensation of rotation will be perceived. Thus there is a latent period of τ sec. From equation (xiv) follows

$$\xi_{min.}=\alpha\frac{\Theta}{\Pi}\tau \quad \text{or} \quad \alpha\tau=\xi_{min.}\frac{\Pi}{\Theta}. \tag{xvi}$$

As the right side of equation (xvi) has a constant value for a certain subject, the product $\alpha\tau$ also has that property, as was first mentioned by Mulder (1908). We propose to call $\alpha\tau$ the 'Mulder product' (Buys & Rylant, 1939; Buys, 1940).

With a sensitive subject:

α (°/sec.^{2})	τ (sec.)	$\alpha\tau$ (°/sec.)
1·0	2·0	2·0
3·6	0·5	1·8
5·0	0·3	1·5

The error in the τ column is rather large for the shorter times, but, nevertheless, $\alpha\tau$ is evidently of the order of 2°/sec.

More may be said of the $\alpha\tau$ product, for from equation (vi) it appears that

$$\alpha\tau = \gamma_{\text{min.}}. \tag{xvii}$$

As these results were obtained from approximated equations, it is to be expected, in this case, that $\alpha\tau$ will be larger than $\xi_{\text{min.}}$, but it remains true that $\alpha\tau$ must have a fixed proportion to $\gamma_{\text{min.}}$.

After infinite time (in practice after about 30 sec.) equilibrium between the directional momentum of the cupula and the inertia of the endolymph will be attained. The cupula is then at rest with a deviation ξ_∞, and

$$\xi_\infty = \alpha\frac{\Theta}{\Delta} \quad \text{or,} \quad \text{as} \quad \frac{\Theta}{\Delta} = 1, \quad \xi_\infty = \alpha. \tag{xviii}$$

This means that the numerical value of the cupula deviation should be equal to that of the acceleration, but only after infinite time. We were able to confirm that the maximal sensation is indeed attained at about 30 sec. As physiological movements last for 3 sec. at most this numerical equality is never reached. This is hardly surprising, because the function of the semicircular canals is to control bodily movements by indicating the attained angular velocity and the angle of rotation executed.

As a certain subjective angular velocity corresponds to a certain deviation of the cupula, the problem is twofold: (*a*) to establish the correspondence between cupula deviation and sensation of rotational velocity; (*b*) to prove that during a physiological movement the sensation of angular velocity is equal to the velocity attained at that moment and that the angle covered by the movement corresponds to the subjective estimation.

(*a*) Some subjects are able to estimate the angle covered by their sensation of angular velocity after the turning test. An angle commonly stated is 360°, or one turn. When the time to cover this angle is measured, the mean subjective angular velocity during this time can be obtained. The subject mentions the moments when he has completed his subjective turns and the observer times them with a stop-watch (Groen & Jongkees, 1948). An example of this kind of experiment is given in Fig. 4. The impulse administered was 40°/sec. and the subjective angular velocity extrapolated to $t=0$ gives 40°/sec., a very good agreement.

In Fig. 4 the slope is again Π/Δ. Here $\Pi/\Delta = 10$ sec.

It is evident that the subjective velocity is equal to the objective one γ, connected to the cupula deviation ξ by

$$\xi = \gamma \frac{\Theta}{\Pi} \quad \text{or} \quad \xi = \tfrac{1}{10}\gamma, \text{ for human subjects,} \tag{xix}$$

and that the determined Π/Δ corresponds to that established in Fig. 2.

The numerical value of the cupula deviation is one-tenth of that of the impulse.

There is not always such a thorough agreement between objective and subjective γ, but sensitive subjects give many results with such agreement, so that the statement is probably true. When there are deviations, they mostly tend to too high a subjective velocity.

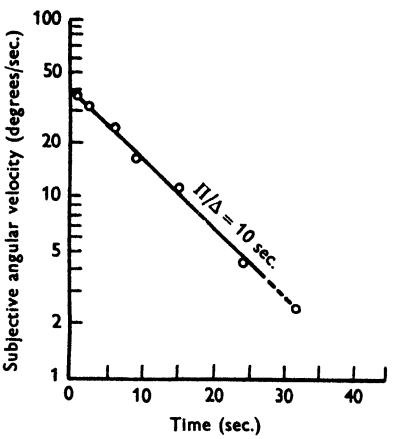

Fig. 4. Subjective angular velocity against time. The impulse administered was 40°/sec., as is the extrapolated subjective velocity. The slope gives $\Pi/\Delta = 10$ sec.

(*b*) The physiological movements consist mostly of an accelerative and a following decelerative phase. The maximal velocity is reached between these two phases. The statement is now that the cupula deviation ought to give rise at any moment to a sensation of rotation equal to the acquired objective velocity. From equation (xvi) it is already known that during the first part of a movement with constant angular acceleration the cupula has a deviation proportional to $\alpha\tau$, otherwise the threshold values of the sensation would never give rise to the constancy of the $\alpha\tau$ product. As $\gamma = \alpha\tau°$/sec. the cupula should have a deviation proportional to γ. This proportionality factor was determined to be $\frac{1}{10}$. Within the limits of the validity of the Mulder product $\alpha\tau$, the theory mentioned above may be generalized for non-constant accelerative movements:

$$\xi \to \gamma = \int_0^\tau \alpha\, dt, \tag{xx}$$

where $\xi = \frac{1}{10}\gamma$ and τ is the moment considered.

In the decelerative phase of the movement the cupula deviation is abolished, but only within the period of validity of Mulder's law, for the attained maximal velocity is reduced to zero, and when the cupula deviation remains proportional to the velocity they decrease together.

If there is a space of time with constant angular velocity between the accelerative and the decelerative phase, the cupula tends to recover zero deviation during this period, with the result that at the end of the decelerative phase the cupula is forced past its zero position and that an after-sensation is caused (clinical turning test, prolonged waltz in the same direction). The conditions imposed by nature on the semicircular canal are therefore confirmed by the theory.

Too short a movement has an opposite effect, i.e. an after-sensation in the same direction as the original movement (in contrast to the results from the clinical turning test). An example of this is the turning of the head.

Turning of the head. This movement lasts so short a time that the $\alpha\tau$ law has not begun to apply when the head has already covered a considerable angle. The Mulder law then comes into action and for the remainder of the movement applies rather strictly. When the head stops the cupula lags behind (a 'fault' originating from the slow start of the cupula) and there is a slight after-sensation for a very short time owing to the peculiarities of this movement. An example of a turning of the head may illustrate this statement. Suppose the head turns from right to left over an angle of 90° in 0·6 sec. The acceleration generally follows a sine function, as may be shown by measurements. The head obeys the equations:

$$\left.\begin{aligned} \ddot{x} &= \alpha \sin \omega t = \omega^2 A \sin \omega t, \\ \dot{x} &= \omega A(1-\cos \omega t), \\ x &= A(\omega t - \sin \omega t - \pi). \end{aligned}\right\} \qquad \text{(xxi)}$$

The angle of 90° gives: $A = \frac{90}{2\pi} = 14°$ approx., $t = 0{\cdot}6$ sec., thus $\omega = 10$ sec.$^{-1}$.

The differential equation of the cupula endolymph is

$$\ddot{\xi} + \frac{\Pi}{\Theta}\dot{\xi} + \frac{\Delta}{\Theta}\xi = \alpha \sin \omega t, \qquad \text{(xxii)}$$

with the limiting conditions at $t=0$, $\ddot{\xi} = \dot{\xi} = \xi = 0$

$$\xi = \frac{\alpha}{\omega\sqrt{(\omega^2 + \Pi^2/\Theta^2)}} \sin(\omega t - \phi) + \alpha \frac{\omega(\Theta/\Pi)}{\omega^2 + \Pi^2/\Theta^2}(e^{-\Delta t/\Pi} - e^{-\Pi t/\Theta}) + \alpha \frac{\omega(\Pi/\Theta)}{\omega^4 + (\Pi\omega/\Theta)^2}.$$

and

$$\tan \phi = \frac{\omega \Pi}{\Theta(\omega_0^2 - \omega^2)} \text{ (approx.).} \qquad \text{(xxiii)}$$

With $\omega = 10$ sec.$^{-1}$, $\alpha = 1400°/\text{sec.}^2 = \omega^2 A$, $\Pi/\Theta = \Pi/\Delta = 10$ sec.$^{-1}$, and $\Delta/\Theta = 1{\cdot}0$ sec.$^{-2}$ the result may be simplified to

$$\xi = -7\,[\sqrt{2}\sin(10t + 45°) + e^{-10t} - 2] \text{ degrees.}$$

The behaviour of the cupula deviation ξ with time is shown in Fig. 5. There is a slow start with the result that the maximal cupula deviation is reached after the middle of the movement. At the end there still remains a deviation of 7°, which is neutralized in about 0·3 sec., for the cupula not only has a deviation, but also a velocity which drives it to zero deviation in accordance with the equation

$$\xi = 7e^{-10t} \text{ degrees.}$$

To test the law of Mulder it is sufficient to consider the behaviour of the cupula in relation to the velocity of the head. At a maximal velocity of the head of 280°/sec., the maximal deviation of the cupula is 24°, corresponding to a subjective angular velocity of $10 \times 24 = 240°$/sec. There is thus a slight discrepancy between objective (280°) and subjective (240°) angular velocity, but in general it may be said that the law of Mulder is valid.

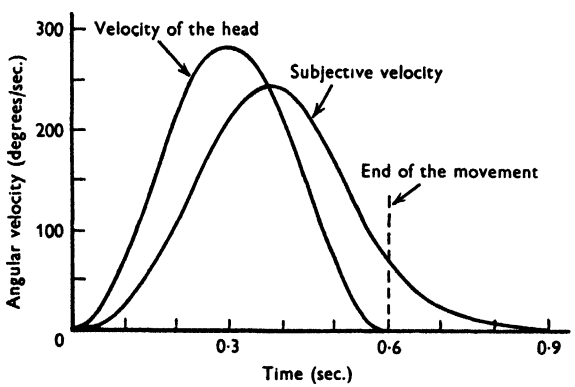

Fig. 5. Turning of the head 90° in 0·6 sec. The angular velocity is maximal at $t = 0·3$ sec. and attains the value 280°/sec. The subjective velocity lags behind, but reaches almost the same value, 240°/sec.

The ballet dancer. When pirouetting a ballet dancer will far surpass the period of Mulder's law. When coming to a standstill the dancer would be very dizzy and control of the situation would be impossible if his head had followed the continuous turning of his body. However, he divides the total turning into parts, keeping his head at rest by looking at a fixed point, and when his body, turning on beneath his head, goes too far he jerks his head to the next halting point. Every jerk with the short rest after it lies within the range of the law of Mulder, so that he minimizes the tendency to dizziness during the turning. This practice is now plausible from the theoretical point of view.

Test 2

When the torsion swing vibrates with a gradually decreasing amplitude, the test subject will have a declining sensation. Ultimately he will only feel the

maxima of the swinging. Then $\sin(\omega t+\phi)$ in equation (x) will be unity (Fig. 6). Thus

$$\xi_{\text{min.}}=\frac{\alpha_{\text{min.}}}{\omega}\frac{\Theta}{\Pi}=\omega A_{\text{min.}}\frac{\Theta}{\Pi}\text{ (approx.).} \qquad \text{(xxiv)}$$

This result, compared to equation (xvi), gives

$$\gamma_{\text{min.}}=\alpha\tau=\omega A_{\text{min.}}=\xi_{\text{min.}}\frac{\Pi}{\Theta}\text{ (approx.).} \qquad \text{(xxv)}$$

With equation (xxv) there is a check for the three results:

(*a*) $\gamma_{\text{min.}}$ determined by experiment and graphically.
(*b*) $\alpha\tau$ determined by experiment.
(*c*) $\omega A_{\text{min.}}$ determined by experiment.

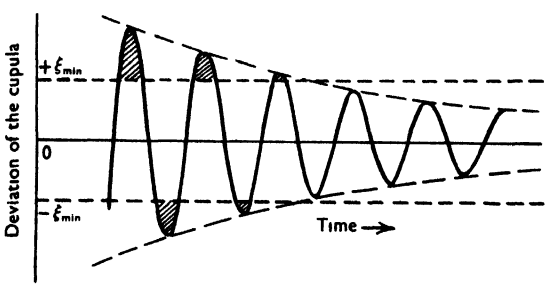

Fig. 6. Deviation of the cupula on the torsion swing in the neighbourhood of the minimum. Only the tops of the declining sine wave are associated with sensation.

As the torsion swing gives an easy controllable sensation, the $\omega A_{\text{min.}}$ measurements may be trusted far more than the $\alpha\tau$ values, which are difficult to obtain:

Period of swing (sec.)	Frequency of swing (sec.$^{-1}$)	Sensation with regard to swing	$A_{\text{min.}}$ (degrees)	ωA (°/sec.)
5·8	1·08	Goes ahead	1·3	1·4
5·0	1·25	Equal	0·8	1·0
4·0	1·57	Lags behind	0·7	1·1
			average	1·2

These results were obtained in a very sensitive subject. For him, $\gamma_{\text{min.}}=1{\cdot}1°$/sec., $\alpha\tau=1{\cdot}5°$/sec. and $\omega A=1{\cdot}2°$/ sec.

His natural frequency $\omega_0=1{\cdot}25$ is higher than usual (1·0) but it is, nevertheless, a common value. On the other hand, there are sensitive persons with $\omega_0=0{\cdot}8$ sec.$^{-1}$. Thus far the theory contains no contradiction.

Test 3

It ought to be possible to administer, directly after the end of the clinical turning test, an angular acceleration which keeps the cupula in its deviated position, from which it would have retreated to zero in the case of a single impulse. The impulse throws the cupula to

$$\xi_{\text{max.}}=\gamma\frac{\Theta}{\Pi},$$

and an adequate acceleration keeps it there. Then

$$\xi = \alpha \frac{\Theta}{\Delta}$$

and

$$\alpha \frac{\Theta}{\Delta} = \gamma \frac{\Theta}{\Pi} \quad \text{or} \quad \frac{\gamma}{\alpha} = \frac{\Pi}{\Delta} \text{ sec.} \tag{xxvi}$$

In practice an acceleration is chosen and the impulse is adapted to it. The subject has the sensation of increasing, constant or decreasing angular velocity when the impulse is too small, well chosen (equation (xxvi)) or too large respectively.

The best way to determine equality is to find two adjacent γ values, one of which is too small, the other too large. A sensation of constant angular velocity is not sufficient, for it might be due to a faulty observation. An example of this experiment may be given:

Angular acceleration (α°/sec.^{2})	Impulse (γ°/sec.)	Sensation	$\frac{\text{Impulse}}{\text{Acceleration}} = \gamma/\alpha = \Pi/\Delta$
3·7	40	Grows	$\frac{35}{3{\cdot}7} = 10$ sec. approx.
	36	Constant	
	30	Decreases	

This test confirms the value of Π/Δ as of the order of 10 sec.

Test 4

The nerve responses of the semicircular canal of the ray. An unexpected proof of the theory has arisen from the measurements of Löwenstein & Sand (1940*a*, *b*). They cut the nervus ampularis of the horizontal canal in the ray and by applying a micro-electrode to one of the fibres of the crista end of the nerve, they measured the frequency of the nerve responses as a function of the applied stimulus. Every fibre has its own resting frequency. When the cupula is at rest, there is apparently a permanent nerve activity which is possibly the origin of the permanent tone originating from the labyrinth. The frequency increases when the canal is stimulated adequately (Ewald) and decreases in the opposite case. Less than zero frequency is not possible and the sensitivity of the canal should therefore be different for opposite directions. In the neighbourhood of the equilibrium position (zero deviation) of the cupula, the sensitivity will be equal for both directions, but not for larger deviations. In Fig. 7 the impulse frequencies given by Löwenstein & Sand are plotted logarithmically with the resting frequency subtracted from the measured frequency. The result is a straight line with a slope Π/Δ of 10 sec., as was the case with the human subject. A resting activity of 30 discharges/sec. was assumed here in contrast to the value which Löwenstein & Sand measured (i.e. 25/sec.).

It follows that something must have happened to the cupula-nerve mechanism as a result of the impact of the endolymph. It is most probable that this rise (or fall) has existed from just after the impact.

As Löwenstein & Sand did not make use of a torsion swing, the determination of the unknown constants has to be derived from equation (iii). From their measurements it may be deduced that the moment when $\xi_{max.}$ is reached should be about 0·6 sec. Thus $t_{max.} = 0{\cdot}6$ sec. From equation (iii) it follows that Π/Θ has to be about 10 sec.$^{-1}$ so that $\Delta/\Theta = 1$ sec.$^{-2}$. The differential equation becomes

$$\ddot{\xi} + 10\dot{\xi} + \xi = 0,$$

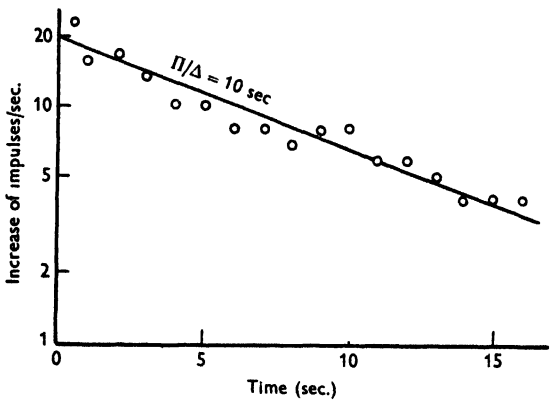

Fig. 7. Response of a single receptor unit of the left horizontal canal to ipsilateral rotation at a constant speed of 36°/sec. Ordinate logarithmic. Replotted from Lowenstein & Sand 1940*b*), after subtraction of resting frequency of 30/sec. from observed frequencies.

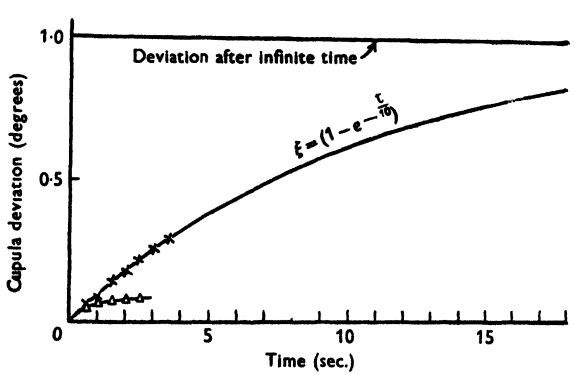

Fig. 8. As Fig. 3, using the figures of Lowenstein & Sand (1940*b*) for the ray. ×, ipsilateral; △, contralateral stimulation. The former agrees well with the theoretical curve, but the latter deviates considerably.

which is the same as in a human subject. This is not surprising since the dimensions of the canals of man and ray are almost the same. The probable error is about 30% for both constants. The uncertainty of Π/Θ is the larger as Θ/Δ could be determined with a reasonable accuracy. The uncertainty is therefore imposed by the Π/Θ factor.

Löwenstein & Sand gave their test animals (the ray) constant acceleration for a short period and recorded the nerve impulses. In Fig. 8 a mean value of twelve measurements is plotted. It is reduced to an acceleration of 1°/sec.^{2}, a procedure which is permissible, because it alters nothing of the essentials of the measured quantities. As far as the measurements allow us to compare, there is no difference between theory and experiment. The contralateral stimulation is also given. It deviates from the ipsilateral curve except for a small region at the beginning. We may therefore conclude that the ray follows the theory of the torsion pendulum for ipsilateral stimulation and for contralateral to a limited extent.

Theoretical value of Π/Θ

When the anatomical data concerning the semicircular canal are known, the value of Π/Θ may be calculated. The moment of inertia Θ is governed by the

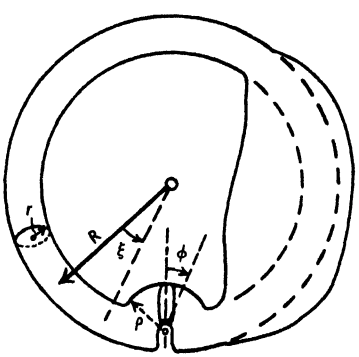

Fig. 9. Schematic diagram of the semicircular canal. When the endolymph moves over an angle of ξ the cupula is forced over an angle of ϕ; ξ and ϕ are of the same order of magnitude.

endolymph ring, including the part in the utriculus (Fig. 9) and the diameter of the cross-section of the ring is determined by the canal itself. Then Θ is given by

$$\Theta = 2\sigma\pi^2 r^2 R^3,$$

where R is the radius of the canal and σ the density of the endolymph. Π, the moment of friction at unit velocity, is derived from Poiseuille's law. As the friction is dictated by the canal and not by the utriculus, the length of the damping canal will be about half the circumference of the circle, πR. The quantity of fluid flowing through a canal with a circular cross-section, radius r, length πR, under influence of the constant pressure P, the viscosity of the endolymph being η is

$$V = \frac{r^4 P\tau}{8\eta R}$$

during the period τ. When the angular velocity is 1 rad./sec. the quantity of fluid is

$$V_1 = \pi r^2 R.$$

The pressure needed is

$$P_1 = \frac{8\eta\pi R^2}{r^2}.$$

The momentum (pressure × surface × radius) is

$$8\eta\pi \frac{R^2}{r^2} \pi r^2 R = 8\eta\pi^2 R^3.$$

This is the moment of friction at unit angular velocity Π.

Thus $$\Pi = 8\eta\pi^2 R^3.$$

From these values follows $$\frac{\Pi}{\Theta} = \frac{4\eta}{\sigma r^2}.$$

As $\eta = 0{\cdot}006$ (c.g.s.) at 37°, $r = 0{\cdot}03$ cm. and $\sigma = 1{\cdot}0$ approx., $\Pi/\Theta = 27$ sec.$^{-1}$ approx.

Such a high value is never obtained with normal persons. As was to be expected there is a slight difference between the original mathematical basis and the experimental results. The most obvious assumption is that there is a leak between cupula and ampulla, an assumption which is confirmed by results obtained from insensitive subjects, who suffer from a large leak or from such symptoms as suggest a leak. Where such a defect exists, the theory has to be modified. The theory thus revised hints at deviations from the ideal case with a close-fitting cupula, the very deviations which are found in practice in insensitive subjects, where there is no close-fitting cupula, but a degree of leak in every ampulla. The cupula-endolymph system is neither a pure torsion pendulum nor a Mach-canal. Sensitive subjects have almost perfectly fitting cupulae and the insensitive less well-fitting ones. A large leak means a gap of about 0·1 mm. at most, whereas a close-fitting cupula has a clearance of not more than 0·01 mm.

Artificial leak. The cupula is only a weak valve. It may be deformed by a small force, i.e. an impact of the endolymph during the clinical turning test. It will then (*a*) permit an increased leak, and (*b*) tend to regain its shape.

(*a*) The artificial leak may be demonstrated by comparing the slope of the graph of subjective angular velocity against time before and after a large impulse. In Fig. 10 impulses of 45°/sec., 180°/sec. and 45°/sec. have been administered to the subject; thus the first and last are of the same magnitude. The second impulse has deformed the cupula, causing it to leak. This is expressed in the smaller $-\Pi/\Delta$ value (2·8), whereas Π/Δ is 6·2 before deformation. Where the deformation is not too severe, the process of restoration will last only 3 hr. In severe cases it will take weeks, or even months before the original value is regained. This has been observed by us in several cases. This is why impulses of not more than 60°/sec. average have usually been used in this work. In sensitive subjects they do not exceed 45°/sec. and in insensitive cases 90°/sec.

As initial test impulse, 30°/sec. is used. A sensation lasting about 20 sec. may then be expected for sensitive persons, whereas 6 sec. is a common value for insensitive ones. The result then obtained in the unknown subjects decides on the procedure to be followed.

(*b*) The deformation of the cupula is in most cases only temporary. There is some reason to believe that the process of restoration begins immediately after the deformation.

The impact of the endolymph deforms the cupula so as to compress one side of it whilst the other is overstretched. In consequence, the sensory hairs terminating in fine canals in the cupula on one side retreat from the canal wall and on the other are pressed further into the canals. The recovery of the cupula restores the hairs to their original attitudes by a series of successive stretchings, and so brings about secondary after-sensations. These have nothing to do with a normal after-sensation which follows an exponential law. The curling of the cupula does not follow a fixed law; nor do the secondary after-sensations. Usually they are alternate, but sometimes two consecutive sensations have the same direction. This is a strong argument for the assumption of the peripheral origin of these secondary after-sensations. Furthermore, the secondary after-sensations occur only when the cupula is deformed (after impulses > 60°/sec., not after smaller ones). In addition, secondary after-sensations only occur with sensitive subjects. All secondary after-sensations are not peripheral, though most are.

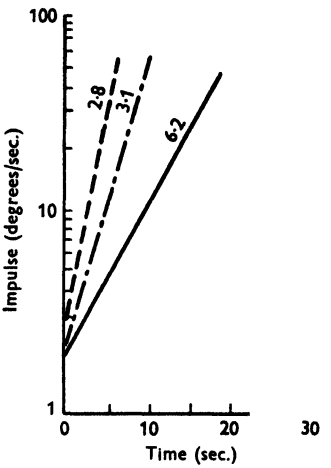

Fig. 10. Time-course of sensation before and after Bárány test, showing slow restoration of cupula. There is considerable diminution of sensitivity even after 4 days. —— before; — — — 1 hr. after; —·— 4 days after.

The foregoing arguments have led to the abolition of the Bárány test in our clinic, a test which may be said to yield at best values concerning a deformed system and never any information on the original physiological properties of the observed canal.

SUMMARY

The semicircular canal with its cupula-endolymph system is best considered as a torsion pendulum with a high degree of damping. There is always a slight leak between cupula and ampulla, which causes deviations from the theoretical behaviour described by the solution of the differential equation of the torsion pendulum. It is possible to calculate the movements of the cupula, taking the leak into account, and the results then correspond to those obtained from insensitive subjects. It is not asserted that the degree of leakage decides

whether a subject is sensitive or not, but it is a likely explanation; though central causes cannot be excluded.

The calculations show that the semicircular canal acts as an 'impulsometer'. For physiological movements it is a most appropriate instrument, comparable to a flux-meter (ballistic galvanometer) used for the measurement of electric charges. It normally causes no after-sensation. Excitation of the canal gives rise to a very accurate subjective estimate of the angle of turning, provided that the movement lasts not more than 3 sec. (Mulder's law). If a turn lasts too long or has a period of constant velocity, the cupula no longer acts as an impulsometer, but is forced back to equilibrium by its own directional property. A decelerative phase following one of constant velocity not only annihilates the deviation, but drives it past equilibrium in the opposite direction, from which the cupula will slowly return, giving rise to an after-sensation. In the clinical turning test this is done intentionally.

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EFFECT OF DOSAGE ON THE RATE OF DISAPPEARANCE OF ETHANOL FROM THE BLOOD OF DOGS

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In 1919 Mellanby, investigating the disappearance of ethanol from the blood stream of dogs after oral administration, came to the conclusion that 'whatever the amount of alcohol in the body, the rate of oxidation is constant'. This was generally accepted, in spite of its being a most unusual behaviour for a metabolite, until controverted by Haggard & Greenberg who, in 1934, published curves of blood-ethanol concentration following intravenous injection of ethanol in the dog. These curves were of exponential form, indicating a rate of metabolism proportional to dosage. Nobody before or since has secured such results, nor have the authors risen in their defence. However, the work was provocative of further investigation of the problem. Newman & Cutting (1935), by means of constant intravenous infusion in man, showed that, with fairly low concentrations of ethanol from 15 to 94 mg./100 c.c. of blood, the amount of ethanol required to maintain a given concentration was independent of the concentration. Neymark & Widmark (1936) published additional curves of blood-ethanol concentration in the dog which had a straight line form, reaffirming the constant rate thesis.

However, in 1937, Newman, Lehman & Cutting reported findings which showed some effect of dosage on rate of ethanol metabolism, after intravenous administration in the dog. In this work they covered a much greater range of concentrations than they had in the previous work with human subjects, using doses of 1, 2, 4 and 6 c.c./kg. They found that, in this range, for each doubling of the dose there was an increase in rate of about 17 %, far less than that reported by Haggard & Greenberg (1934), but still unmistakably significant. In spite of this effect of concentration they felt that the individual curves retained their straight line form, a seeming paradox for which they could offer no satisfactory explanation.

Eggleton, in 1940, re-opened the investigation of the problem with intravenous injection of ethanol. She used cats anesthetized with pentobarbitone. Her results showed that, in accord with the earlier work of Newman *et al.* (1937), the rate of metabolism was higher at higher concentrations, although in no wise proportionally as maintained by Haggard & Greenberg. However, she found that the blood-ethanol curves departed from the straight line form, being less steep as the concentration lessened. Further, she demonstrated that constant infusion of ethanol, at a rate which caused the blood-ethanol concentration to increase when this was at a low level, was insufficient to maintain a high level of concentration without decline.

Clark, Morrissey, Fazekas & Welch in 1941 showed that, in the dog, higher rates of ethanol metabolism were associated with higher dosage, but their published curves show the straight line form, as do also those of Gregory, Ewing & Duff-White in 1943. Thus, it seemed evident at this juncture that there was unanimity of opinion that larger doses of ethanol are metabolized at higher rates, but some divergence regarding the constancy of the rate of decline of blood-ethanol concentration after a single dose. Newman and co-workers offered, as a possible explanation for their findings, that the rate of fall of blood ethanol was in some way conditioned by the highest concentration reached. This cannot be said to explain the results; it does little more than restate their findings in different terms. Because of this apparent divergence of evidence, it was deemed advisable to investigate the problem further.

METHODS

Adult dogs of both sexes were used, without anesthesia. Ethanol was injected intravenously by gravity as a 20% solution in an isotonic solution of sodium chloride. The rate of injection never exceeded 0·5 c.c./kg./min., and for the continued maintenance of an already attained concentration was much less than this. Administration of small divided doses by stomach tube was resorted to for very prolonged maintenance. Samples of blood were secured from leg veins at appropriate intervals and analysed for ethanol by the method of Newman & Abramson (1942).

RESULTS

Thirty-nine single injections were made in ten dogs, using doses of ethanol ranging from 0·75 to 3·0 g./kg. The results are summarized in Table 1. The rate of metabolism is expressed in two ways in this table. First, by extrapolation of the best-fitting line between the points representing ethanol concentrations in the blood at varying intervals after injection to the time base, the length of time that alcohol remained in the blood stream could be estimated. Dividing the dose by this time gave the rate of metabolism in mg./kg./hr. This estimation assumes that the disappearance of ethanol from the venous blood indicates the completion of its metabolism in the body. Secondly, by taking the blood-ethanol concentration about 45 min. after the completion of the injection and subtracting from it the concentration at a subsequent time, preferably when

this had approached but not actually reached zero, and dividing the difference by the time elapsed between the two determinations, the rate of fall of blood-ethanol concentration in mg./c.c./min. was obtained. As can be seen from the table, there is quite a constant relationship between the two values, so that little advantage can be claimed for one method over the other, and they are both set down mainly for ease in comparison with the figures of other workers, some of whom use one and some the other means of expression.

TABLE 1. Relationship of dosage to rate of metabolism of ethanol

Dog	Weight	Dose ethanol (g./kg.)	Rate of metabolism mg./kg./hr. *A*	Rate of metabolism mg./c.c. blood/min. *B*
BR	18	3·0	174	0·00364
1	23	3·0	113	0·0024
		1·5	102	0·00223
		1·5	98	0·0021
		1·5	103	0·00228
		0·75	91	0·00222
2	21	3·0	146	0·00288
		3·0	138	0·00257
		1·5	102	0·00203
		1·5	123	0·00252
		1·5	107	0·00237
		0·75	111	0·00218
3	18	3·0	136	0·00308
		1·5	130	0·00273
		1·5	105	0·00233
		1·5	94	0·00218
		0·75	91	0·00213
4	18	0·83	101	0·00237
		0·83	98	0·00213
		0·83	116	0·00233
5	10·5	1·43	102	0·00243
		1·43	104	0·00228
		1·43	106	0·00238
6	14	1·07	133	0·00305
		1·07	133	0·003
		1·07	143	0·00333
A1	17	3·0	156	0·00365
		1·5	122	0·00307
		0·75	103	0·00287
		0·75	91	0·00265
A5	22·5	3·0	95	0·00197
		3·0	101	0·00242
		1·5	82	0·0019
		0·75	71	0·00213
		0·75	83	0·00188
A6	15	3·0	133	0·00312
		3·0	145	0·00297
		0·75	77	0·00223
		0·75	67	0·00177

A, mg./kg./hr., determined by time required for alcohol to disappear from the blood. *B*, rate of fall of blood alcohol concentration in mg./c.c./min.

TABLE 2. Rate of ethanol metabolism after varying dosage. Where more than one trial of a dose was made, the average of the trials is recorded

	Rate of metabolism (mg./kg./hr.)		
Dog	Dosage (0·75–1·07 g./kg.)	Dosage (1·43–1·5 g./kg.)	Dosage (3·0 g./kg.)
BR	—	—	174
1	91	101	113
2	111	111	141
3	91	110	136
4	105	—	—
5	—	104	—
6	136	—	—
A1	97	122	156
A5	77	82	98
A6	72	—	139
Average	98	105	137

Table 2 utilizes the same data, with the rate of metabolism for all trials of similar dosage in each animal averaged. It demonstrates the variability in rate of metabolism at the same dose in different dogs, with, however, a definite increase in rate with increasing dosage.

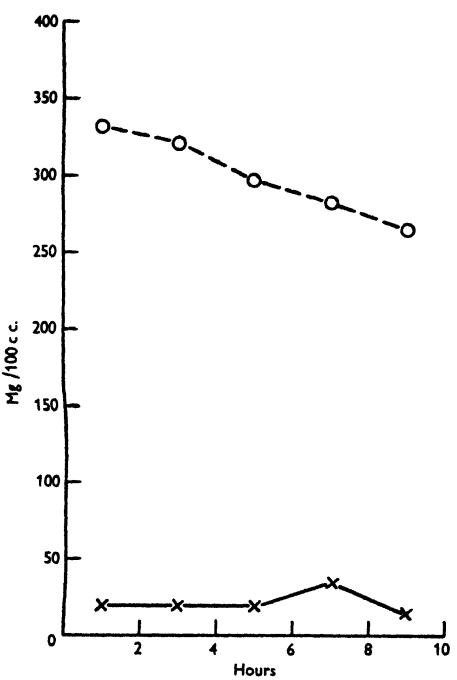

Fig. 1. Blood-ethanol concentration over an 8 hr. period in dog A6, during which period hourly doses of 72 mg./kg. were administered by stomach tube. In the case of the upper curve, an initial dose of 3·0 g./kg. preceded the maintenance dosage, while with the lower curve the initial dose was 0·375 g./kg.

In dog A6, which showed the greatest variability in rate of metabolism with dosage of any of the animals tested, observations were made on the amount of ethanol required to maintain the blood ethanol level constant at differing levels. An initial intravenous dose of 0·375 g./kg. produced a blood-ethanol concentration of 20 mg./100 c.c. at the end of 1 hr. At this time, and every hour thereafter for a total of eight doses, the dog was given a dose of ethanol of 72 mg./kg. by

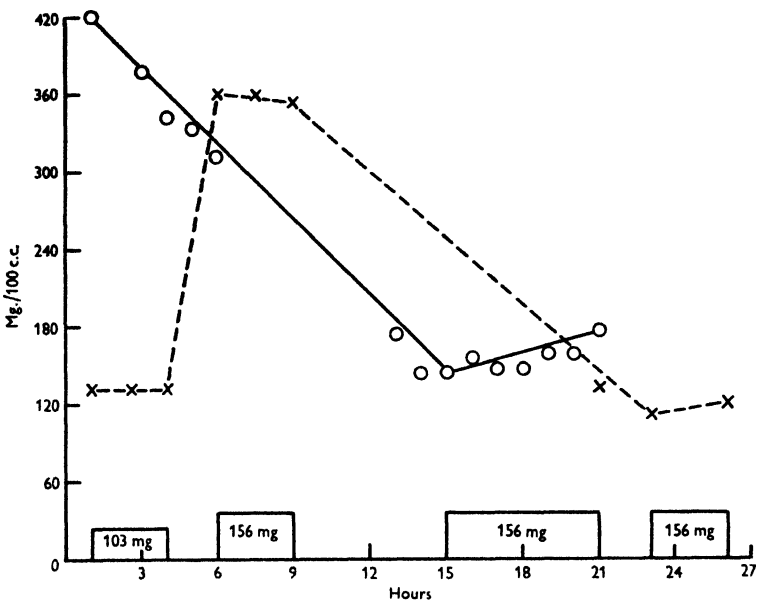

Fig. 2. Curves of blood-ethanol concentration in dog A1. An initial intravenous dose of 0·75 g./kg. was given, and after an hour a constant infusion at the rate of 103 mg./kg./hr. was continued for 3 hr. Then a second large dose was given to raise the concentration to 360 mg./100 c.c., at which level the larger maintenance dose of 156 mg./kg./hr. for 3 hr. failed to maintain this concentration. The concentration was then allowed to fall to a lower level, and the same maintenance dose of 156 mg./kg./hr. resulted in a definite rise in concentration in 3 hr. In a second trial, shown in the solid line, the maintenance dose of 156 mg./kg./hr. was continued for 6 hr. after the concentration had been allowed to drop from an initial value of 420 mg./100 c.c., and again a very definite rise in concentration resulted.

mouth, this being its estimated rate of ethanol metabolism at low blood-ethanol levels. As can be seen from Fig. 1, this dosage was successful in maintaining the blood-ethanol level very nearly constant for the 8 hr. period. On a subsequent day the same procedure was repeated, except that the initial dose of ethanol was 3·0 g./kg., giving a concentration at the first hour of 330 mg./100 c.c. In this instance the hourly doses of 72 mg./kg. were inadequate to maintain the blood concentration constant at this high level, and a fall of 65 mg./100 c.c. in the 8 hr. resulted.

In dog A1, which also showed a considerable effect of dosage on rate of ethanol metabolism, an initial intravenous dose of 0·75 mg./kg. resulted in a blood ethanol concentration of 132 mg./100 c.c., as seen in Fig. 2. This was maintained very nearly constant for a period of 3 hr. by a constant intravenous infusion at the rate already found for this level of blood-ethanol concentration in this dog, 103 mg./kg./hr. At the end of the 3 hr. period the blood-ethanol concentration was raised by a second rapid infusion to 362 mg./100 c.c., and again the constant infusion started, this time at the rate previously found for an initial dosage of 3·0 g./kg., namely 156 mg./kg./hr. This proved to be inadequate to prevent a slight drop in concentration over the 3 hr. period, the blood-ethanol concentration falling to 352 mg./100 c.c. No further ethanol was then given for 14 hr., by which time the concentration had fallen to 112 mg./100 c.c. Then a constant infusion was again started, at the same high rate of 156 mg./kg./hr., with the result that the blood-ethanol concentration rose in 3 hr. to 120 mg./100 c.c. On another occasion, in the same animal, an initial dose resulting in a concentration of 420 mg./100 c.c. was given, as seen in the solid line in Fig. 2. The concentration was allowed to decline to 144 mg./100 c.c., and then the constant infusion at the rate of 156 mg./kg./hr. started. At the end of 6 hr. the concentration had risen to 176 mg./100 c.c.

DISCUSSION

The results set forth in Table 1, and summarized in Table 2, demonstrate the degree of variability in the rate of metabolism of ethanol in different dogs at the same dosage level. Equally is shown the definite increase in rate when the dosage is increased from the vicinity of 0·75 g./kg. to 1·5 g./kg. and finally to 3·0 g./kg. Since the average blood ethanol value for the entire period that ethanol remained in the blood stream after the 0·75 g./kg. dose was about 50 mg./100 c.c., and that after the 3·0 g. dose was 200 mg./100 c.c., it is seen that the 40% increase in rate of metabolism brought about by this increase in average concentration of 150 mg./100 c.c. is not far out of line with the 30% increase for an increase of 100 mg./100 c.c. reported by Eggleton. We have thus confirmed her work, and incidentally our own previous experiments.

Reference to Table 1 shows also the relatively constant rate of metabolism for a given dose in a given dog. The reduction in rate at lower concentrations is much more apparent in some animals than in others. Thus an increase in dose from 0·75 to 3·0 g./kg. caused an increase in rate of metabolism of 93% in dog A6 and only 24% in dog. 1.

The data presented in Fig. 1 demonstrate that the same divided dose of ethanol at frequent intervals which is capable of maintaining the blood-ethanol level constant at low concentrations is incapable of so doing when the concentration in the blood is at a high level. This confirms the observation of Eggleton, and extends it to the unanaesthetized dog.

Newman and co-workers (1935, 1937, 1942), while demonstrating the increase in rate of ethanol metabolism with increasing dosage, nevertheless found difficulty in reconciling this finding with the apparent straight line form of their curves of blood ethanol. They presented the hypothesis that the rate of ethanol metabolism was in some way conditioned by the highest concentration to be reached in the blood, so that this high rate characteristic of high dosage would be maintained even when the concentration fell during the course of metabolism. Eggleton showed that a dose of ethanol administered by infusion at a constant rate which was more than capable of maintaining a low blood-ethanol concentration was inadequate for this purpose when the blood-ethanol concentration was subsequently raised to a high level, and this has been confirmed by our present work. However, this demonstration does not of itself invalidate the hypothesis of 'conditioning' put forth by Newman and co-workers, since the low concentration was the first tested, and thus the 'conditioning' by a preceding high level had not had a chance to take place. However, if the hypothesis were correct, it should be possible to maintain the blood-ethanol concentration constant at a low level by the constant infusion of a dose of ethanol equal to that required at a much higher level, if the constant infusion was given after the blood-ethanol level had been raised to this high level initially, and then allowed to drop to the desired low level before the constant infusion was begun. The results of such an experiment are shown in Fig. 2. This shows conclusively that a constant infusion of ethanol at a rate slightly less than that required to maintain the blood-ethanol concentration constant at a high level will result in an increase in blood-ethanol concentration at a lower level, even though this lower level has been reached by decline from a much higher one. We must, therefore, accept the fact that under all circumstances ethanol is metabolized at a slower rate at low concentrations than at high, and that the apparent straight line form of the published curves of a number of workers, including ourselves, must be explained by the finding that the rate of fall of blood-ethanol concentration has sufficient irregularity to obscure the relatively small decrease in rate of metabolism with declining concentration.

CONCLUSIONS

The rate at which ethanol is metabolized in the dog bears a relationship to the concentration of the alcohol in the animal body, the higher the concentration the higher the rate. This relationship is not, however, a strict proportionality. It varies considerably from one animal to another. The relatively small degree of the effect of dosage, and the inherent irregularity of disappearance of alcohol from the blood are sufficient to account for the apparent straight line form of the published curves of concentration against time.

SUMMARY

1. The rate of decline of ethanol concentration in the blood of dogs after intravenous injection of varying doses was determined. The amount of ethanol per unit time required to maintain the blood-ethanol concentration constant at varying levels was also found.

2. There is a definite relationship between dose and rate of ethanol metabolism, a 40% average increase in the latter resulting from an increase in blood-ethanol concentration of 150 mg./100 c.c.

3. In view of these findings, the apparent linearity of blood ethanol curves in published work must be due to the relatively minor effect of dosage on rate of decline, coupled with the inherent irregularity in the rate of disappearance of ethanol from the peripheral blood.

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THE MECHANICAL ACTIVITY OF SINGLE MOTOR UNITS IN REFLEX CONTRACTIONS OF SKELETAL MUSCLE

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In studies of the activity of skeletal muscles, more attention has been given to the electrical than to the mechanical responses of the individual motor units, and the original electrical techniques of Adrian & Bronk (1929) and of Denny-Brown (1929*a*) have been widely used. A general picture of the mechanical properties of a single unit can, however, be inferred from the responses of various whole mammalian muscles, excited electrically (Cooper & Eccles, 1930).

A mechanical record taken from the tendon of a reflexly excited muscle shows no clear evidence of the contributions of the single units taking part in the contraction unless only one unit happens to be active. Eccles & Sherrington (1930) show a record of a short tetanus in a single unit of the cat's soleus made under these circumstances, but the method is perhaps too exacting to have wide application in the investigation of single units, because it can only be used with minimal responses. In a previous paper (Gordon & Holbourn, 1948*b*) we have described a method of recording the mechanical and electrical responses of single motor units in small human muscles: this method isolates the contraction of one unit in a muscle where many other units may be active, by recording from a very small area on the surface of the muscle. The method depends upon recording the sideways swelling of the fibres, and in principle it resembles the technique used by Denny-Brown & Pennybacker (1938) for investigating muscular fibrillation. The method has now been modified for recording the longitudinal movements of single units from the surface of exposed muscles in the cat, and in this paper we describe the activity of single units in the reflexes of stretch, crossed extension, and ipsilateral flexion. A preliminary description of one part of this work has already been given (Gordon & Holbourn, 1948*a*).

METHODS

Cats, decerebrated at intercollicular level, were used for the experiments. The decerebration was done under deep ether anaesthesia, and 2–4 hr. were afterwards allowed for the effects of the anaesthetic to wear off. All the animals breathed spontaneously, and had a well-marked extensor

rigidity. In experiments on spinal flexion reflexes, the spinal cord of a decerebrate animal was subsequently divided at L1, giving a spinal hindlimb preparation which did not need to be artificially ventilated. A further small amount of ether was given just before dividing the cord. The muscle from which the record was to be made (usually crureus or tibialis anterior) was exposed by dissection, and separated to some extent from its neighbours so as to give access to as much of its surface as possible: it was kept in good condition by applying paraffin and warm Ringer-Locke solution to the surface. The rectal temperature was recorded with a thermocouple.

Reflex contractions of deep extensor muscles (e.g. crureus) were elicited, in the decerebrate animal, either by passive stretching of the muscle (stretch reflex) or by pinching the toes of the opposite foot (crossed extension reflex). Contractions of flexor muscles (e.g. tibialis anterior) were elicited by pinching the toes on the same side (spinal flexion reflex).

Recording apparatus. Two alternative forms of apparatus were used for recording the movements of single motor units on the surface of a muscle. In each, a pair of wire electrodes was incorporated for picking up muscle action potentials.

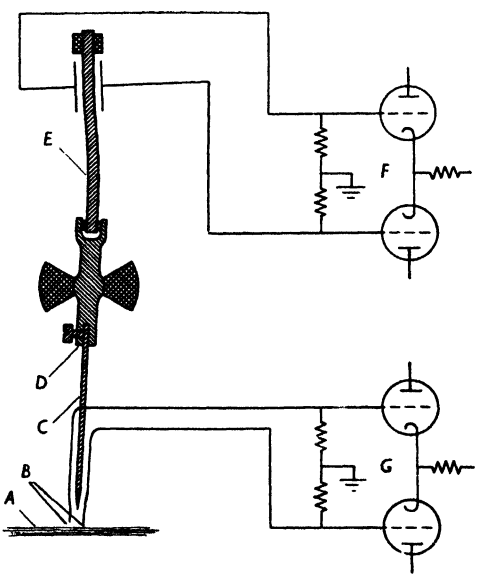

Fig. 1. Piezo-electric instrument (for explanation, see text).

In the first type (Fig. 1), longitudinal movements of the muscle were picked up by a metal rod (*C*) inserted in the chuck (*D*) of a Rothermel piezo-electric gramophone pick-up ('bender' type), the output of the crystal (*E*) being led through screened leads to an amplifier (*F*). The time-constant of the whole system was 60 msec. The action potentials were picked up by a pair of constantan wire electrodes (*B*), attached to the sides of the metal rod with their ends projecting 2 mm. beyond the lower end of the rod on to the muscle (*A*). The electrodes were connected through screened cable to a second amplifier (*G*). In this way, the records of movements and action potentials were made simultaneously from the same small area of the muscle (inter-electrode distance 1 mm.). The pickup could be moved in relation to the muscle by a rack-and-pinion adjustment. The piezo-electric pick-up was calibrated in terms of tension and displacement by the methods described by Sandow (1944): the curve relating tension to electrical response is exponential, but the relation between displacement and tension at the myograph point is approximately linear, the movement being 8μ for each g. of tension. This movement is so small that the myographic records made with this instrument can be considered virtually isometric. Unfortunately the short time-constant of

the apparatus leads to some distortion of the recorded movement, so that the rising phase of a twitch is the only part which is faithfully reproduced.

The second type of apparatus was a very sensitive miniature torsion myograph (Fig. 2), the torsion strip (*C*) being made of beryllium-copper (0·038 mm. thick, 0·98 mm. wide, and 12 mm. long). A rod, 10 mm. long, was soldered perpendicularly to the strip, so as to project on either side of it, one projection carrying a thin mirror (*B*, 3·5 × 2·5 mm.) and the other carrying a pair of wire electrodes (*A*) which projected a little beyond the end of the rod on to the muscle. These electrodes (0·16 mm. diameter, 0·2–0·5 mm. separation) were connected by an 8 cm. length of 0·09 mm. diameter copper wire to a pair of terminal tags (*D*), which were also connected to the input of an amplifier. In recording from this mirror myograph, the conventional arrangement of a slit-source of light and a cylindrical lens was used, the mirror being about 1 m. away from the recording paper.

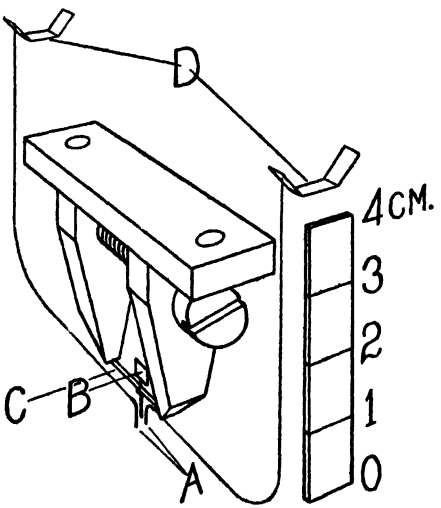

Fig. 2. Mirror myograph (for explanation, see text).

With this arrangement, a 25 mm. (full-scale) deflexion on the paper corresponded to 0·5 g. tension or 87 μ displacement at the myograph point. It can be seen that this myograph allows considerably more movement than the piezo-electric instrument. The natural frequency of the myograph with the electrodes and their connecting wires attached was 160 cyc./sec., which is high enough not to introduce any appreciable distortion into records of the twitches of limb muscles.

When making records with either of these instruments, the animal was laid on its back in a moulded 'Celastic' trough, which supported its body, hips and thighs. A twist-drill was driven through the condyles of the femur and rigidly clamped to the animal stand to increase this fixation. The limb distal to the muscle under observation was allowed to remain free, so that some shortening was allowed; but it will be seen from our records that the shortening of an individual motor unit, with the myograph in position, was quite small.

RESULTS

If the surface of an exposed muscle is kept moist and suitably illuminated, it is possible to see minute shimmering movements taking place there during reflex contraction. If crureus is watched during a slight progressive stretch of the patellar tendon, the vibratory movements start in one or two areas of the

surface, but more and more areas become involved as the stretch is increased. The movements represent the subtetanic contraction of isolated single motor units, since they occur in time with the rhythmical action potentials recorded from the same areas.

These movements and their corresponding action potentials can be recorded simultaneously by applying a myograph, of a kind already described, to the surface of an active region of the muscle. The myograph is pressed on to the surface until the electrode points, which are disposed in line with the direction of the muscle fibres, just catch in the fascial investment of the muscle. In crureus, the area from which the movements of a single unit can be recorded is usually a longitudinal strip about 1 mm. wide and 1 cm. or so in length. In tibialis anterior, the strip is often much smaller than this. Good records from single units can usually be obtained, in the slowly recruited extension reflexes, in a muscle where a number of units are active, provided that the motor units immediately adjacent to the one under observation are not contracting. In the *d'emblée* opening of the flexion reflex, however, it is very difficult to achieve the isolation of a single unit.

It is clear that with this method of surface recording, one will only be picking up mechanically from the most superficial of the fibres belonging to any motor unit, so that the tension of any recorded contraction will be considerably smaller than the tension developed by the whole unit. One would expect to record a force nearer to the total tension in the unit when the myograph was applied to a point on the muscle nearer to the tendon end. The position is further complicated by the fact that any active unit is bound by connective tissue to other parts of the muscle, so that its free movement is restricted. Probably this effect is small in experiments, like those to be described, where little shortening of the muscle was allowed to take place.

The characteristics of single twitches of single motor units in different muscles

A single motor unit, under the influence of very slight reflex excitation, gives single isolated twitches. Under good experimental conditions, a record shows that contraction and relaxation in the unit are represented by a smooth curve, without any sudden changes of gradient (Fig. 3*a*). In this respect the twitches resemble those obtained from whole muscles in the cat by Cooper & Eccles (1930). Sometimes the electrical activity of another unit, probably lying deeper in the muscle, is recorded without any evidence of its mechanical response; in this case one may still record the twitches of a superficial unit simultaneously without much distortion (Fig. 3*b*). Records of the twitches of a single unit are, nevertheless, apt to be deformed by the activities of other units more or less remote from the myograph point, the result being an irregular distortion which is seen clearly in Fig. 3*d*, a record taken during a crossed extension reflex in crureus.

It is well known that muscles differ very widely in the time which they take to develop maximal tension in a twitch (Denny-Brown, 1929*b*). Cooper & Eccles (1930) described this characteristic of a muscle in terms of 'contraction-time', which was measured from the beginning of the action potential to the peak of the ensuing twitch. We have found it more convenient to measure from the origin of the twitch to its peak, which should give a figure which is shorter than the contraction-time only by the period of latency. The latency is, in fact, so short (less than 2·5 msec.) that our measurement of time-to-maximum should be comparable with the index of Cooper & Eccles.

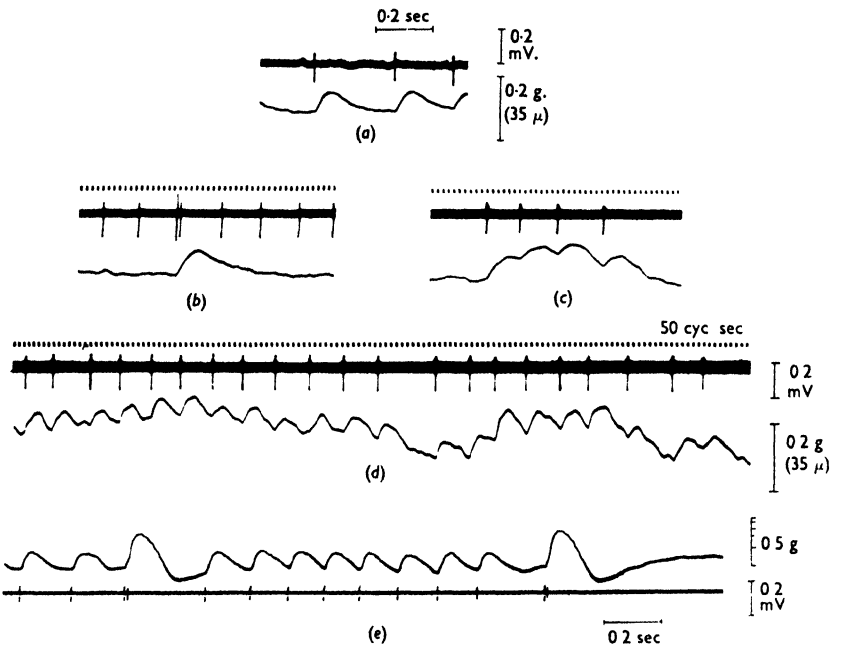

Fig. 3. Simultaneous records of action potentials and contractions from single motor units of crureus in the decerebrate cat. (*a*) Crossed extension reflex. Action potentials on top trace, mirror myograph record on bottom trace. Rectal temp. 37° C. (*b*) Crossed extension reflex. Arrangement of record as in (*a*). (*c*) Tonic stretch reflex. Arrangement of record as in (*a*). (*d*) Crossed extension reflex. Arrangement of record as in (*a*). Rectal temp. in records (*b*) to (*d*), 35° C. The calibrations shown after (*d*) refer to records (*b*), (*c*) and (*d*). (*e*) End of crossed extension reflex, showing two double discharges of the unit. Piezo-electric myograph record on top trace, action potentials on bottom trace. Rectal temp. 37° C.

Measured in this way, the time-to-maximum for single twitches was 60·9 msec. (35 observations, S.E. of mean ± 1·18) in crureus, 28·9 msec. (15 obs., S.E. ± 0·65) in the subcutaneous fibres of tibialis anterior, and 62·6 msec. (15 obs., S.E. ± 1·63) in the units on the deep and tibial surface of tibialis anterior. Our figure for crureus, which is a red muscle, is less than the contraction-time given by Cooper & Eccles (1930) for the whole soleus (94–120 msec.), but crureus is said to be

a more rapid muscle than soleus, although both are slow (Denny-Brown, 1929*b*), which partly explains the discrepancy.

Our two figures for tibialis anterior are interesting, because in most cats the subcutaneous surface of this muscle is considerably paler in colour than the deep surface. When one compares the rate of tension development in units from the pale and red surfaces, it becomes clear that tibialis anterior is a muscle in which quick and slow units are wedded together, both taking part in the flexion reflex. This arrangement of superficial quick muscle and deep slow muscle is well known to be a feature of extensor muscle-groups (Denny-Brown, 1929*b*), but has not been described in flexors.

The subtetanic contraction of single motor units

Single isolated twitches are not often seen in a single unit during reflex contractions unless the stimulus is minimal. The usual activity seen in stretch reflexes (Fig. 3*c*), crossed extension reflexes (Fig. 3*d*, *e*) and flexion reflexes

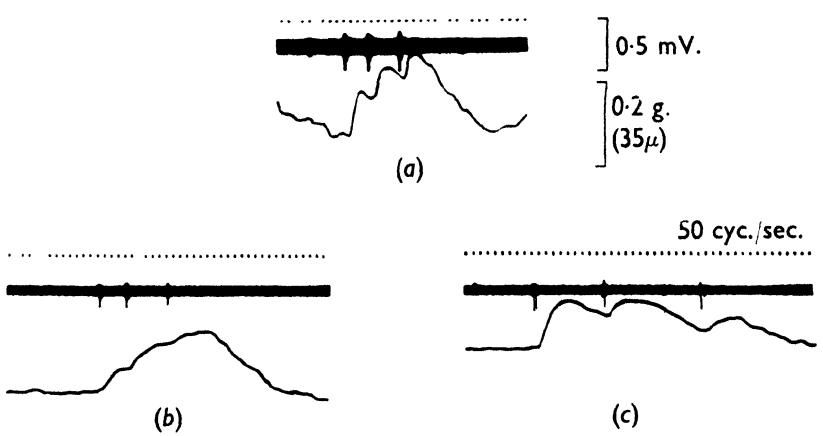

Fig. 4. Simultaneous records of action potentials and contractions from single motor units of tibialis anterior in the decerebrate cat (cord cut at L1). Rectal temp. 37–36° C. (*a*) Record taken from the subcutaneous surface of the muscle during a flexion reflex. Action potentials on top trace, mirror myograph record on bottom trace. (*b*) Record from the deep and distal surface of the muscle during a flexion reflex. Arrangement of record as in (*a*). (*c*) As (*b*) above. A record from the same unit, which now gives an initial double discharge.

(Fig. 4) is subtetanic, in which a greater or lesser degree of fusion exists between the individual twitches. The amount of fusion of individual twitches which occurs at any particular frequency quite clearly depends upon the 'quickness' or 'slowness' of the unit concerned, and fusion would occur more easily in crureus than in the pale part of tibialis anterior. On the other hand, the frequency of firing in the extension reflexes of crureus is usually so much lower than it is in the pale part of tibialis anterior during a flexion reflex that single units in these muscles seem to fall short of complete fusion by about the same

amount (cf. Figs. 3*c*, *d* and 4*a*). In the same way, the frequency of firing in units on the surface of tibialis anterior is higher than in the slow units of the deep part of the muscle: the highest frequency we have seen in the quick part was 50/sec. and in the slow part 28/sec., the contraction being elicited by pinching the toes. No single motor unit, in our experiments, has ever been seen to reach a state of complete fusion, a fact which may partly be attributed to the need for keeping the stimulus fairly small.

It is sometimes possible to record from a strip of the muscle surface upon which two motor units are pulling simultaneously, and giving twitches of approximately the same size. In Fig. 5, the two units are firing asynchronously, and can be recognized by the difference in the size of their action potentials; at one point they happen to fire almost together, so that the potentials are partially summed. In this record one can see the beginnings of the summation process which makes the contraction of the whole muscle smooth if a large enough number of units are firing asynchronously.

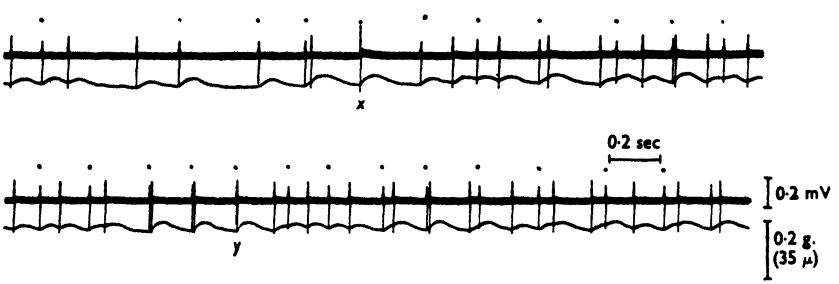

Fig. 5. Two sections of a record from two simultaneously active motor units of crureus during a crossed extension reflex ('tonic appendage'). Decerebrate cat, rectal temp. 37° C. Action potentials as top trace, mirror myograph record on bottom trace. The action potentials of one of the two units are marked, for identification, by circular dots. At *x*, the two units fire almost synchronously, and their potentials are partially summed: at *y*, they fire about 3 msec. apart.

Double discharges in single motor units.

In reflex firing of a single motor unit, it is common for the motor neurone to give one or more double discharges, especially in stretch and crossed extension reflexes when the neurone is firing very near to its threshold (Adrian & Bronk, 1929; Denny-Brown, 1929*a*; Eccles & Hoff, 1932; Hoff & Grant, 1944). These double discharges are found most commonly at the beginning of a contraction, less often at the end. The interval between the two spikes of a double discharge lies between 2·9 and 20 msec. (Hoff & Grant, 1944), and the second spike, when recorded from the muscle, is usually somewhat smaller and of greater duration than the first. The fact that the rhythm of discharge of the motor neurone is altered and slowed when a double discharge is given, is evidence that the second impulse is not due to the firing of another motor unit (Eccles & Hoff, 1932).

The mechanical response of the motor unit to a double discharge is shown in Fig. 3*e* and Fig. 4*c*. The result appears to be a complete fusion of two twitches, and the total tension developed is rather more than twice the tension of a single twitch. The ratio between the tensions in a single and in a double discharge must depend partly on the initial tension of the muscle fibres, which we have not controlled. It will also depend to some extent on the capacity of the fibres to give a second full-sized response after such a short interval, but the results of Eccles & O'Connor (1939) on stimulation of the whole soleus suggest that there would be no appreciable reduction in total tension until the interval was reduced to about 2·3 msec.

The effect of an initial double discharge can be seen by comparing Fig. 4*b*, where a unit in the slow part of tibialis anterior starts its burst of activity with a single impulse, and Fig. 4*c*, where the same unit starts with a double discharge. It is clear that the double discharge allows a large initial development of tension, which could then be maintained by a fairly low frequency of firing.

Double discharges are found quite often in crureus and in the slow part of tibialis anterior, but we have not seen them in the quick part of the tibialis. It is possible that they are absent or rare in quickly contracting muscle, but we have not examined a large enough number of different muscles to feel certain that this is so. Denslow (1948) has found that double discharges are more common in some human muscles than in others, though he did not attempt to relate their occurrence to any particular property of these muscles.

DISCUSSION

It has already been pointed out that the method which we have described for recording the contractions of single motor units does not give a measurement of the tension which the whole unit is developing. Our records, when very little shortening is allowed, have never shown more than 0·6 g. tension in the twitch of a single unit of crureus, which is of the order of one-quarter of the probable value for the whole unit given by Eccles & Sherrington (1930). This must be very much more than the value for a single muscle fibre, however, and our results suggest that at least a considerable number of the fibres in a motor unit, in the muscles which we have examined, are lying closely together in a muscle bundle. This agrees with the conclusion of Denny-Brown & Pennybacker (1938).

The method does, on the other hand, give a fairly satisfactory picture of the time course of the contraction, even when a number of other motor units are active in the same muscle. This has allowed us to compare the 'contraction times' of units in different parts of the same muscle, with consistent results, and this particular type of investigation could not easily be made by any other means. There is no reason to believe that tibialis anterior is alone among the flexor muscles in containing fibres with widely differing contraction times, and there is room for a more complete investigation of this point. To do this in

a satisfactory way, our present technique would have to be modified, in order to control the temperature of the muscle more accurately. The units from which records have been made were superficial, and therefore apt to be cooler than the rest of the muscle. Temperature has an important effect on the rate of contraction, and in some preliminary experiments with a muscle enclosed in a warm chamber the contraction time for the quick part of tibialis anterior has been found as short as 19 msec. at 38° C. (Gordon & Phillips, 1949).

The fact that completely fused tetanic contractions of single motor units have not been seen has been attributed partly to the small stimuli which were used. At the same time, we have measured the highest frequencies which occurred in electrical records with strong stimuli, and it is clear, from the relation between fusion frequency and contraction time given by Cooper & Eccles (1930), that fusion could not have occurred at these frequencies. Perhaps the only occasion on which complete fusion normally occurs in a single motor unit is during the summed twitch which follows a double discharge of the neurone. An initial discharge of this sort establishes a very high initial level of tension, and has been seen in both flexion and extension reflexes. In a spinal reflex, an initial double discharge in some motor units might partly explain why the first few steps of a reflex tetanus are often larger than those of a motor nerve tetanus in the same muscle (Liddell & Sherrington, 1923). Double discharges are not peculiar to spinal and decerebrate preparations; they have been found by Gilson & Mills (1941) and by Denslow (1948) as normal forms of activity in human muscles. The initial double discharge might provide an alternative mechanism to the *d'emblée* entry of a number of motor units, or it could give additional impetus when a *d'emblée* contraction occurred. Denslow (1948) has pointed out that the prolonged repetition of double discharges which he has seen in the human trapezius must maintain a high level of tension in a tonic contraction.

Finally, it is as well to emphasize the practical limitations of the method of recording described. Mechanical records can only be made from the most superficial motor units, and even then the record will be adequate only if the unit under observation is of fairly low reflex threshold. If the reflex threshold of the unit is high, then a large number of other units, both remote and adjacent, will already be contracting by the time this unit comes into action, and the record will be distorted. It is also important that the unit should not have an immediate neighbour which is also superficial and which has a similar reflex threshold, because the two units will both pull on the myograph, like those recorded in Fig. 5. In the presence of these difficulties, it is clear that any unit, from which an adequate record is to be made, needs careful selection. At the same time, this selection is not unduly difficult, and the scope of the method, in its present form, is indicated by the experimental results we have described.

SUMMARY

1. A method, and two forms of myograph, are described for recording simultaneously the contractions and the action potentials of single motor units from exposed muscles in the cat. The merits and shortcomings of the method are discussed.

2. The average contraction-time of single unit twitches in crureus was 60·9 msec. It was found that the contraction-time of the units on the subcutaneous surface of tibialis anterior (28·9 msec.) was much shorter than that for the units on the deep and distal surface of the same muscle (62·6 msec.). It is concluded that this flexor muscle contains a mixture of quick and slow muscle fibres.

3. Complete tetanic fusion of the twitches of a single motor unit was not seen in reflex contractions, except when the motor neurone gave a double discharge. In this case the tension rises smoothly to a level which is rather more than twice the tension of a single twitch. The phenomenon of the double discharge is briefly discussed.

The authors would like to express their thanks to Mr Peter Philbin and to Mr P. V. Skinner for their help with the experiments.

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A PREPARATION OF SURVIVING RAT SMALL INTESTINE FOR THE STUDY OF ABSORPTION

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Despite the physiological importance of the activities of the intestinal mucosa, there have been few successful attempts to study it in isolation, and the impression has arisen that it is difficult or impossible to set up a satisfactory surviving intestine preparation apart from the body and still with a viable mucosa. The objects of this paper are to show that this is not so, to describe a preparation which can be set up with uniform success, and to describe its major properties.

In outline, the preparation to be described is made by cannulating at both ends a segment of small intestine in an ether-anaesthetized rat, and setting up a closed circulation through its lumen of an oxygen-saturated, CO_2-bicarbonate-buffered fluid *before* the circulation through the intestinal wall is interrupted. The segment is suspended in a bath of oxygenated Ringer, and absorptive processes are followed by sampling the fluid circulating through the lumen (inner fluid) and the fluid bathing the exterior of the intestine (outer fluid).

The success of the technique is believed to depend on the feature that there is no time at which the mucosal cells are deprived of an adequate supply of oxygen.

METHODS

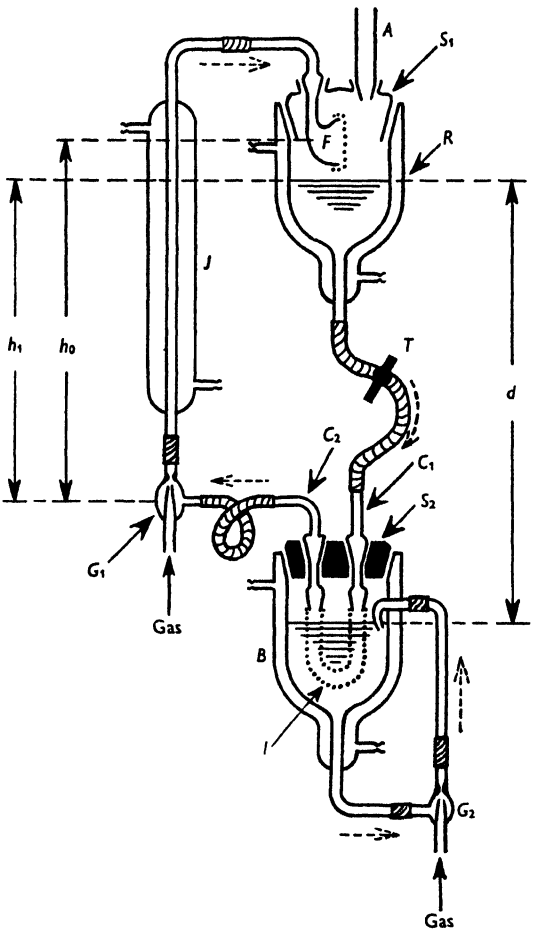
Animals. Male albino rats of Wistar stock weighing 200–300 g. are used. The animals are taken off the stock diet 24 hr. before use, and are provided with plain water and 5% glucose in water. They usually take about 100 ml. of the glucose-water, so that their calorie intake is reasonably maintained, but at the same time the small intestine is rendered largely free of solid contents.

Anaesthesia. Induction is effected by placing the animal in a small box containing a cotton-wool pad on which ether has been poured. Light anaesthesia is maintained with an Oxford Vaporizer adapted for use with small animals.

Preparation of segments. Two segments from each animal are usually set up, the whole of the small intestine except the duodenum being used. The duodenum has been excluded in the work so far carried out because of difficulties in mobilizing it, and for fear of harmful traction on the blood vessels or the intestine.

The abdomen is opened in the midline, the duodenal-jejunal flexure identified, and the intestine divided at this point. A cannula directed caudad is tied into the jejunum, the length of upper

segment required is decided, the intestine is again divided at the appropriate place, and the upper cannula of the lower segment is tied in. The upper segment is now washed free of debris with a gentle stream of warm 0·9 % NaCl directed caudad, and a second cannula is tied into its lower end. (If the lower cannula is tied in earlier, it tends to block during washing.)



Text-fig 1. Circulation unit; for explanation see text.

The operating table, which is mounted on a trolley, is now brought up to one of the 'circulation units' described below, in which the inner and outer fluids are maintained at 38° C. and are kept oxygenated. The upper segment cannulae are connected into the inner fluid circuit and circulation is started in the direction from upper to lower end of the segment. The mesentery of this segment is now clamped and divided peripherally to the clamp, the freed segment is dipped into 300–400 ml. of warm saline to remove traces of blood, and is then set up in the outer fluid bath. The procedure for the lower segment is the same. The time taken, from completion of induction of anaesthesia to setting up of the second segment, averages 10 min.

Circulation units. Text-fig. 1 shows diagrammatically the layout of a circulation unit. The inner fluid passes from the double-walled glass reservoir R through a rubber tube furnished with a screw-clamp T to the glass tube C_1. Half-way along C_1 is a standard glass cone, and at the lower end

there is a smaller standard cone. The upper cone seats in a tapered hole in the ebonite stopper S_2. The inner fluid passes from C_1 through the loop I to a second similar glass tube C_2. At the beginning of an experiment I is a glass U-tube, connected by short lengths of rubber tubing. Later it is replaced by a segment of intestine. The fluid passes from C_2 to G_1, which is a small gas-injector which introduces a stream of gas bubbles into the liquid flowing through it. The inner fluid then passes via the jacketed vertical tube J and the antifrothing device F back to the reservoir R. F is a small hemispherical glass bowl with a short side tube emerging tangentially at the bottom of the bowl. The opening of the bowl is covered by cheese-cloth impregnated with paraffin-wax. This device is held by a short length of rubber tubing on to an angled glass tube fitting into a standard tubulure in the glass stopper S_1 of the reservoir R. S_1 carries a second standard tubulure into which fits the air condenser A.

When gas is injected through G_1 the mean density of the fluid in the portion G_1JF of the inner circuit falls to a value much below that of the fluid in the portion RTC_1IC_2, so that fluid is forced to circulate in the direction shown by the dotted arrows so long as gas is supplied to G_1 and T is open. The pressure differential determining the rate of flow is $(h_1 - ah_0)$, where h_0 and h_1 are the heights indicated on Text-fig. 1, and a is the fraction of the volume of the upflow tube which is occupied by liquid. With $h_1 = 35$ cm., $h_0 = 40$ cm. and tubes of *c.* 4 mm. internal diameter, inner fluid circulation rates of 35–45 ml./min. are obtained.

The distance d in Text-fig. 1 is equal to the difference, in terms of head of water, between the pressures inside and outside the intestine, i.e. is the distension pressure. Some circulation units have been made up in which the distance d can be varied without affecting the pressure differential determining the rate of flow.

The outer fluid passes from the double-walled bath B through a vertical tube at the bottom of the bath to a gas-injector G_2 and thence, through a vertical return tube, back into the bath by way of a horizontal tube sealed through its walls. The gas introduced into the bath by the injectors is carried away by an air-condenser (not shown in the figure) fitting into a third tapered hole in the stopper S_2.

Water is circulated through the jackets of B, J and R at high speed from a thermostatically controlled reservoir maintained at 39° C. With a circulation rate of 4 l./min./unit, there is less than 0·05° C. difference between the inner fluid temperatures of six units heated in parallel, and there is no detectable variation of temperature with time. The temperature of the inner and outer fluids is maintained at 38° C. by this arrangement. The set of six circulation units normally used is mounted on a rack designed for rapid dismounting of components for cleaning, and supported on a stand so that the baths B are at the height of the mobile operating table.

Setting up of intestinal segments on circulation units. With the inner circuits completed by glass U-tubes at I, 50 ml. of the appropriate inner fluid are introduced into each unit through S_1, and 50 ml. of the appropriate outer fluid are introduced into B through S_2. T is opened and 5% CO_2 in O_2 is introduced through G_1 and G_2, the flow being adjusted to give a regular pattern of gas bubbles and liquid films in the upflow tubes. The inner and outer fluids attain steady temperatures in 5–10 min. from the beginning of circulation.

To set up an intestinal segment, T is closed, S_2 is lifted out of B, and the rubber connexion of the glass U-tube to C_1 is taken off, S_2 being held well above the level of G_1 to prevent back flow of inner fluid. Inner fluid in the glass U-tube and beyond is returned to R by flowing through the U-tube, which is then disconnected from C_2. The intestinal cannulae, which have internal tapers corresponding to the lower cones on C_1 and C_2, are then connected in place of the glass U-tube, being held on by short rubber tubes. The clamp T is opened to start fluid circulating through the intestine, and, after the intestine has been freed and washed, as already described, it is introduced into B and the stopper S_2 replaced. Samples of inner and outer fluids can be withdrawn at any time by means of pipettes introduced through S_1 and S_2.

Composition of circulation fluids. On all experiments, so far, the circulation fluid for both circuits has been basically a Krebs bicarbonate medium (containing 0·0012 M-phosphate), made up according to the directions given by Umbreit, Burris & Stauffer (1945, p. 194), which when equili-

brated with 5% CO_2 in O_2 gives a pH of approximately 7·4. As a general rule 500 mg. glucose/100 ml. has been added to this medium. The inner circuit fluid also regularly contains 1 ml. of 0·3% phenol red/50 ml. Krebs medium. Phenol red does not pass across the intestinal wall in detectable amount in our conditions, so that it provides a convenient way of detecting the minute pinholes which occur on occasion in the neighbourhood of the ligatures securing the intestine to the cannulae.

RESULTS

Histological appearances

Sections of segments of intestine, removed from the animal immediately after cannulating and washing with saline, have been compared with sections prepared from intestine which has survived on a circulation unit for an hour. Pl. 1 illustrates comparable sections of fresh and surviving upper jejunum fixed in Bouin solution and stained with haematoxylin and eosin. It will be noted that in the higher power illustrations there is evidence in all sections of disengagement of cells from the mucosa of the tips of the villi. No distinction in this respect has been found between fresh and surviving intestine. Leblond & Stevens (1948) have recently reported the same phenomenon in normal intestine, and give reasons for supposing that continual replacement of the mucosa is a normal process.

An attempt to assess the magnitude of this mucosal shedding by determining the nitrogen passing into the inner fluid in an hour period of survival gave the answer that there was no detectable addition of nitrogen in five experiments, whilst in a sixth 16 μg. N/cm. length of intestine appeared. This corresponds to 3% of the mucosal nitrogen (mean of eight determinations is 0·5 mg. mucosal N/cm. length intestine, measured on material removed from longitudinally split segments by scraping with a blunt spatula).

Apart from this finding of cellular disengagement, the mucosa appears histologically to be in good order, and there is no indication of the gross stripping usually reported in surviving preparations (Verzár & McDougall, 1936, p. 19).

When the intestinal segment is fixed *in situ* on the circulation unit, which may be done by replacing the greater part of the inner and outer fluids, in that order, by Bouin's solution, and continuing circulation for 30 min., the conformation of the mucosa is very different from that seen in a normal fixed preparation made by dropping the excised segment into fixative. Pl. 1*a* shows the 'normal' appearance, and Pl. 1*c*, *e* show the appearances of segments fixed at different distension pressures. The appearances of Pl. 1*c*, *e* are very like those illustrated by Johnson (1912–13) and they are much more like Johnson's illustration of the conformation of the guinea-pig intestinal mucosa fixed after tying off when the intestine was normally distended with food than is Pl. 1*c*.

One other histological feature which is marked in Pl. 1*c*, *e* is the distension of the submucous space in the surviving segments. Measurements of the water content of comparable segments of small intestine before and after an hour survival period indicate that, with 500 mg. glucose/100 ml. in inner and outer fluids, the water accumulation amounts to 0·5 g./g. initial weight. Although

this amount appears large, it corresponds to only 0·02–0·04 ml./cm. intestinal length.

Transfer of water across the intestinal wall

If water as well as solutes is transferred across the intestinal wall, then the extent of this transfer, as well as the change in concentration of the solute, must be known before estimates of rates of transfer of solute can be made. No substance has so far been found which can conveniently be used for estimating volume changes in the circuits. Phenol red, for example, which is transferred trivially if at all across the mucosa, nevertheless appreciably stains it. The current procedure is to drain both circuits very carefully at the end of the period of observation into 100 ml. graduated cylinders. Estimates of initial volumes are not essential, since, in the absence of a volume change indicator, all that can be observed is the total movement of solute from beginning to end of the period of observation, and the total amount of solute originally introduced into each circuit is known. Initial volumes can be obtained, where necessary, by taking initial samples from each circuit and dividing the concentration of solute in these samples into the amount of solute originally introduced. These volumes are regularly larger than the volumes originally placed in the circuits, because some of the washing saline is inevitably trapped in the lumen and entrained in mesenteric remnants on the serosal surface of the intestine.

Measurements of initial and final volumes made in these ways, with practically identical composition of inner and outer fluids, show that a significant transfer of water takes place (Table 1) from inner to outer circuit, the magnitude

TABLE 1. Movement of water from inner fluid of normal segments of intestine, surviving for 1 hr., with originally identical inner and outer fluids containing 500 mg. glucose/100 ml.

Segment location	Water leaving inner fluid (ml.)	Length of segment (cm.)	Shift per cm. intestine (ml.)
J	10·8	37	0·29
J	10·1	45	0·22
J	12·6	63	0·20
J	8·9	49	0·18
I	8·1	42	0·19
I	7·1	42	0·17
I	6·8	49	0·14
I	4·9	48	0·10

I = ileal, J = jejunal segment.

of the shift being 0·1–0·3 ml./cm. intestine/hr. Since the initial water content of 1 cm. of intestine is about 0·05 ml. the rate of transfer corresponds to the movement of 2–6 times the initial water content of the intestine per hour. It is not surprising, in these conditions, that there should be some water retention in the tissue. The magnitude of the retention (0·02–0·04 ml./cm.) is not such as to suggest that there is any serious barrier to diffusion through the submucosal tissues. On the contrary, it is clear that, in the presence of such a swift water

current across the submucosal region, rates of appearance of solutes in the outer fluid, when corrected for the known extent of water retention in the intestinal wall, may safely be taken as good measures of the rate of disengagement of such solutes from the peripheral border of the mucosa.

Transfer of glucose across the intestinal wall

The transfer of glucose may be used to illustrate this point. Table 2 presents the results of experiments in which changes in glucose content in inner and outer fluids have been measured, the initial concentrations in the two fluids being initially the same, in the region of 500 mg./100 ml. and the survival period being 1 hr.

TABLE 2. The discrepancy between rate of disappearance of glucose from inner fluid and rate of appearance of glucose in outer fluid

Segment location	Inner fluid: disappearance rate (mg./cm./hr.)	Outer fluid: appearance rate (mg./cm./hr.)	Discrepancy
J	2·89	1·05	1·84
J	2·73	1·21	1·52
J	2·20	0·50	1·70
J	2·00	0·63	1·37
I	2·20	1·60	0·60
I	2·06	1·14	0·92
I	1·71	1·11	0·60
I	1·68	1·33	0·35
		Mean	1·11

The discrepancies in the last column of Table 2 are all in the sense to indicate an appreciable loss of glucose in passage across the intestinal wall. Further experiments were made, therefore, in which intestinal segments were maintained in the same conditions on circulation units for the same time, and the intestine killed at the end of this period by rapid disengagement from the circulation unit and plunging into water at 100° C. The total glucose in the intestine plus circulating fluids was then determined. The upper part of Table 3 gives the differences, expressed as mg. glucose, between the amounts of glucose originally introduced and the amounts recovered. Unfortunately, these figures cannot be expressed exactly in mg./cm. intestine, since the intestine contracts on immersion in boiling water. But in view of the variability of the results in Table 2, no more than an order of magnitude is needed for comparison. A column has therefore been added to Table 3 giving estimated glucose disappearance per cm., on the basis that, in our conditions, the intestine contracts on average to 80 % of its initial length.

The possibility that the losses of glucose observed in these last experiments are due to events occurring in the course of killing the intestine has been controlled by similar experiments in which the intestine is removed and killed within 5 min. of setting up on the circulation unit. The results, given in the

lower part of Table 3, indicate that there is no artefact due to glucose destruction during killing of the intestine.

The mean water content per cm. of perfused intestine is 0·08 ml./cm. The mean difference between the discrepancies of Table 2 and the 60 min. utilization given under Table 3 is 0·22 mg. glucose/cm. Thus, if the glucose retained in the intestinal wall is distributed throughout its total water, it must be present in a concentration of 22/0·08 mg./100 ml., i.e. approximately 280 mg./100 ml. Since the final concentrations in the two fluids in contact with the intestine are

TABLE 3. Disappearance of glucose from the whole system, intestine plus inner and outer fluids, after different periods of survival on circulation units

Segment location	Period of survival (min.)	Disappearance of glucose (mg.)	Estimated disappearance per cm. intestine
J	60	55·2	1·21
I	60	37·9	0·83
J	60	47·8	0·94
I	60	6·0	0·22
J	60	52·0	0·88
I	60	16·6	0·62
		Mean	+0·78
J	5	−1·8	−0·03
I	5	−7·1	−0·28
J	5	5·4	0·13
I	5	1·1	0·03
		Mean	−0·04

Algebraic difference of the two means (=0·82 mg./cm.) gives estimated utilization for 55 min. The corresponding figure for 1 hr. is 0·90 mg./cm.

usually both in excess of this figure, the observed degree of retention is consistent with the view that the retained glucose is present in some fraction of the intestinal water, for example, the extra-muscular water, in a concentration approximating to that of the outer fluid. There is certainly no suggestion of any serious barrier to diffusion of glucose across the submucosal tissues.

Effects of degree of distension

In view of the necessity to use some degree of distension to ensure flow through the intestine, and since some authors (e.g. Verzár & McDougall, 1936; Sols & Ponz, 1947) have described effects of distension on absorption from intestine *in situ*, some experiments have been made to examine the effects of different degrees of distension on active glucose absorption from surviving segments.

There is a gradient of activity along the intestine in rate of active glucose absorption from inner fluid, the evidence for which is presented in a subsequent paper (Fisher & Parsons, 1949), the gradient taking the simple form that the uptake of glucose per cm. intestine per hr. is linearly related to the mean distance of the segment (i.e. of the centre of the segment) from the ileo-caecal

valve. Utilizing this gradient, one can compute a 'standardized absorption rate', i.e. the absorption rate that would have been expected had the segment in question come from a standardized location in the small intestine. Table 4 presents the results of experiments in which two segments of intestine from each animal were set up, one at 35 cm. H_2O distension pressure and the other at 10 cm. H_2O, all observed rates being corrected to a standardized mean distance of 40 cm. from the ileo-caecal valve. These results indicate that change in degree of distension is without effect over this range. As, however, segments surviving at 10 cm. distension pressure regularly show peristaltic activity, which is vigorous in jejunal segments, whereas segments at 35 cm. distension pressure are always quiescent (cf. Magee & Southgate, 1929–30), and as the peristalsis may be vigorous enough on occasion to interrupt flow, with a consequent risk of anoxia, 35 cm. H_2O distension pressure is used as a routine.

TABLE 4. Effect of degree of distension on the rate of active absorption of glucose from surviving rat small intestine

Standardized absorption rate at 40 cm. from ileo-caecal valve (mg. glucose/cm. intestine/hr.)

Animal	10 cm. H_2O distension pressure	35 cm. H_2O distension pressure
159	1·67 (I)	1·30 (J)
161	1·50 (J)	1·91 (I)
166	1·56 (I)	1·61 (J)
167	1·79 (J)	1·74 (I)
Means	1·63	1·64

It is unlikely that the peristaltic activity is in any way related to the nutrition of the intestine since it is abolished immediately by raising the distension pressure and reappears when it is lowered.

Effects of anoxia

In early experiments with surviving intestine the mesentery was clamped and cut, and the freed segment washed briefly in saline before it was connected into the inner fluid circuit of the circulation unit. Although these steps were taken as rapidly as possible, there were 20–30 sec. during which the mucosa had little or no oxygen supply. Comparing these experiments with the later ones in which there was no interruption of oxygen supply, there are two obvious differences:

(1) With interrupted oxygen supply the intestine frequently showed vigorous motor activity at 35 cm. H_2O distension pressure, which persisted for all or most of the standard hour period of observation. With continuous oxygen supply motor activity is never seen in these circumstances.

(2) With interrupted oxygen supply the phenol red of the inner fluid regularly indicated a pH shift to the acid side, more marked in jejunal than ileal segments, the pH appearing to drop continuously throughout the period of observa-

tion. With 30–40 cm. jejunal segments (the small intestine of the rat being 110–130 cm. long, of which 15–20 cm. is duodenum) the pH shift might exceed 0·5 unit, the phenol red becoming quite yellow. Such a shift is in conformity with the aerobic glycolysis quotient for rat jejunum quoted by Dickens & Weil-Malherbe (1941), provided that the lactate remains in the inner fluid. No pH shift is observed in the outer fluid.

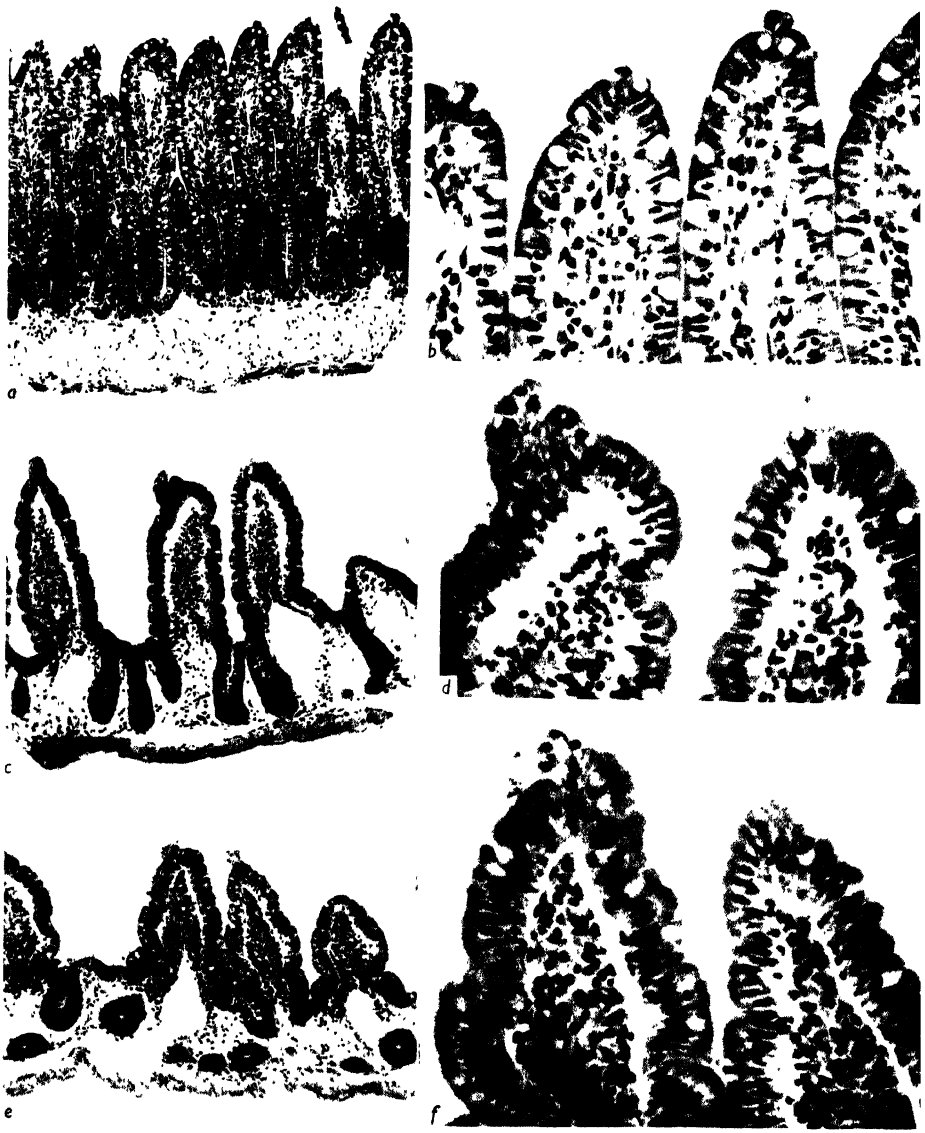
With continuous oxygen supply it is sometimes possible to detect a slight acid shift in the inner fluid, but no such shift as those regularly observed with interrupted oxygen supply has been seen in well over 100 segments of surviving intestine.

Both these observations show that brief anoxia can produce disturbances which far outlast the period of anoxia, and emphasize the importance of our precautions to avoid the briefest anoxia.

DISCUSSION

The one indication of mucosal disintegration observed in the preparation described in this paper parallels a process described in normal intestine by Leblond & Stevens (1948), who describe disengagement of fragments of mucosa at the tips of villi, the remaining mucosa sealing up behind these fragments. They link this process with the constant presence of mitoses in the crypts of Lieberkühn, a phenomenon which has been seen in all our preparations, and they have recently shown, by a radioautograph technique (Leblond, Stevens & Bogoroch, 1948) that there is good reason to believe that the view that mucosa is constantly being replaced at a high rate is correct.

The ability of the intestine to remove glucose from the lumen when the initial concentrations of glucose on the two sides of the intestinal wall are equal, and the active translocation of water, are evidence that the cells of the mucosa of the preparation are capable of active work, but a consideration of the scale of activity is best deferred to a later paper (Fisher & Parsons, 1949). One other piece of evidence for the metabolic activity of the preparation may be cited here. The figures for glucose utilization in Table 3 correspond to Q_{O_2} values ranging from 53 to 10, if it is assumed that the glucose is completely oxidized, an assumption which appears probable since no appreciable accumulation of acid products occurs. These figures may be compared with those of Dickens & Weil-Malherbe (1941) for isolated intestinal mucosa. The activity of their preparations steadily diminished with time, the highest Q_{O_2}'s observed being 31 for jejunum and 11 for ileum. The present preparation thus shows a distinctly higher metabolic rate than the maximal rates in Dickens & Weil-Malherbe's experiments. It is also of interest that these authors observed marked aerobic glycolysis in rat small intestine, which may be linked with the present observation of a marked acid change in surviving intestine exposed to a brief anoxia. It seems unlikely that the metabolic pattern disclosed by tissue respiration



a. Normal jejunum, fixed in Bouin's solution. Haematoxylin and eosin. ×80.
b. High-power view of *a*. ×272.
c. Surviving jejunum, fixed in Bouin's solution at 10 cm. H_2O distension pressure. Haematoxylin and eosi
d. High-power view of *c*. ×272.
e. Surviving jejunum, fixed in Bouin's solution at 35 cm. H_2O distension pressure. Haematoxylin and eosin.
f. High-power view of *e*. ×272.

To face

experiments in which the tissue has been exposed to anoxia to the extent which is usual in such work can safely be interpreted as normal, at least in so far as the small intestine is concerned.

Despite the artificiality inherent in its divorce from the remaining tissues of the body, the surviving intestine has a number of advantages over other preparations used to study intestinal absorption. The Cori (1925) technique has the major disadvantage that the concentration in the intestine and the portion of the intestine effecting the absorption are unknown, and are open to modification by secondary factors such as gastric and intestinal motility (e.g. see Fenton, 1945). The Verzár (1936, p. 18) technique of injecting a solution into a tied-off segment *in situ* has the two disadvantages: (*a*) that maintained anaesthesia is necessary, and (*b*) that, with a readily absorbed substance, the change in concentration in the small amount of fluid that can be introduced is so large that specification of a concentration at which absorption takes place is artificial. The most recent technique, that of Sols & Ponz (1947), in which a solution of the solute to be studied is allowed to flow through a cannulated segment, suffers from the disadvantage that anaesthesia is required. All these techniques have, in addition, the disadvantage that they permit the study of one aspect only of the process of absorption, disappearance of solute from the intestinal lumen. It has already been indicated that a considerable amount of glucose taken up from the lumen may be utilized in the intestinal wall, and it is worth making a rough calculation on the basis of the data of Table 3. Taking the average rate in this table of 0·90 mg. glucose/cm./hr. as applicable to the whole 100 cm. of intestine of a 200 g. rat, the utilization amounts to 0·45 g. glucose/kg. body wt./hr. when the whole small intestine is exposed to 0·5% glucose. This is an appreciable fraction of the usually quoted figure for absorption rate, determined by the Cori technique, of 1 g./kg./hr., and raises important questions concerning the general significance of reported absorption rates.

The work reported in this paper indicates that the new technique described should be capable of analysing movement of solute from the lumen into its components of utilization and translocation. It possesses another advantage over the older methods in that it makes it possible to study the form in which the absorbed material passes from the mucosa into the underlying tissue fluids.

SUMMARY

1. Apparatus and procedure are described for instituting circulation of oxygenated fluids through and around isolated segments of rat small intestine without subjecting the tissue to anoxia during the procedure.

2. The histology of the mucosal cells of the preparation is shown to be essentially normal after survival for an hour.

3. Evidence is provided that the preparation contains actively metabolizing cells, capable of active translocation of solutes across the intestinal wall, and

that there is no appreciable barrier to diffusion across the submucosal tissues of the intestinal wall.

4. The preparation extends the possible range of studies of intestinal absorption, since it renders accessible to study the form and amount in which solutes leave the peripheral border of the intestinal mucosa.

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FACTORS DETERMINING THE TIME COURSE OF RIGOR MORTIS

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One of the most difficult features of rigor mortis to explain is the great variation between one animal and another in the interval between death and the first signs of stiffening in the muscles. In 1930, in respect of twenty rabbits, this was shown to vary without attributable cause. It was suggested in 1939 (Bate-Smith) and again in 1947 (Bate-Smith & Bendall, 1947*a*), that violent activity immediately before death was the main reason for accelerated onset of rigor. The circumstances affecting the time after death of onset of rigor have now been examined in greater detail, and it is apparent that, at a particular temperature, this time is determined by two considerations: the pH of the muscle at the moment of death (itself determined by activity immediately preceding death); and the magnitude of the glycogen reserve of the muscle (which, as is now shown, determines the pH at onset of rigor). The interval between these two pH values is covered by glycolysis, which proceeds at a rate dependent upon pH and temperature, but is otherwise almost invariable from one animal to another.

The two factors concerned have been varied experimentally. Activity preceding death has been controlled by injection of myanesin which causes complete muscular relaxation and abolishes the death struggle (Berger & Bradley, 1947; Bate-Smith & Bendall, 1947*b*). The magnitude of the glycogen reserve has been varied: (*a*) by injection of insulin in convulsant doses, which leads in the extreme limit to complete exhaustion of glycogen; (*b*) by reducing or increasing the intake of food. These various treatments appear to affect the post-mortem behaviour of the muscle only in so far as they affect the factors mentioned, and it is therefore unnecessary in analysing the results to separate the animals treated in a particular way from the animals not so treated. The results, at the given temperatures, form homogeneous groups.

For the most part, observations have been made at room temperature (17° C. approximately). A number of observations have been carried out both at room

temperature and at 37° C., using paired psoas muscles; and a few experiments have been carried out at 37° C., using one of the psoas muscles for chemical determinations and the other for registration of rigor. The effect of temperature is mainly evident in the increased rate of glycolysis, which results in a shorter interval between death and the onset of rigor, and in a marked increase in shortening of the muscle during rigor at the higher temperature.

METHODS

Pre-treatment of the rabbits.

In order to obtain muscles of the desired glycogen content and initial pH three methods were adopted:

(1) *Control of the feeding of the animal.* To obtain intermediate levels of glycogen in the muscle the animals were starved 48–72 hr., and to obtain high levels they were fed on an ample diet of carrots and oats for 2–3 weeks.

(2) *Insulin injection.* In order to obtain animals with virtually no glycogen reserve and an initial pH value of 6·9–7·2 large doses of insulin (up to 100 units) were injected intraperitoneally to produce vigorous convulsions. Five animals were treated in this way.

(3) *Myanesin injection.* 300 mg. myanesin (B.D.H.) per kg. of body weight were injected intraperitoneally to produce paralysis (cf. Bate-Smith & Bendall, 1947*b*). The animals were completely relaxed within 5 min. of the injection and were killed after 30–40 min. In this way initial pH values between 6·9 and 7·2 were always obtained. Twenty-four animals were thus treated. The total glycogen reserves were regulated by method (1).

Other animals used.

Four experiments with bullocks are recorded. These animals were killed by shooting and subsequently bled and decapitated by the ordinary slaughterhouse procedure. The results of forty experiments with rats exercised with varying severity before death are also discussed. The rats were exercised in power-driven drums of 1·5 ft. diameter, revolving at 120 ft./min. They were removed after varying periods and killed immediately by stunning and decapitation.

Measurement of modulus of elasticity (E)

The elasticity of the rabbits' psoas muscles was measured by the method of Bate-Smith (1939), but with the modification that the muscles were automatically loaded and unloaded by means of an electrically operated arm, designed to give a cycle of 8 min. under load, and 8 min. with the load removed, the cycle being repeated continuously until rigor was fully established. The arm was raised and lowered by a taut resistance wire, attached at one end to the extremity of the arm and at the other to a rigid support slightly above the fulcrum. At rest the tension in the wire was sufficient to keep the arm raised when loaded with a weight of 100 g. To lower the arm a current was passed through the wire, sufficient to heat it to about 200° C., thus allowing it to stretch. The current was passed at each loading and shut off at each unloading. Two instruments of identical pattern were available, enabling comparative measurements to be made on the right and left psoas muscles of the same animal. The record was made on a smoked drum of 6 in. diameter, a complete revolution of which took 11½ hr. The recording needle was arranged to give a 7·5-fold magnification of the actual changes of length of the muscle. The type of diagram obtained, illustrated in Fig. 1, has been described in an earlier communication (Bate-Smith & Bendall, 1947*b*).

The modulus is calculated on the rapid portion of the recovery curve, and represents the recovery in ¼ min. This value is referred to as α, and the slow portion (¼–8 min.) as β. This procedure is necessitated by the reduced time-scale of the record, and the values are, therefore, not identical with the A and B values calculated by Bate-Smith (1939) from a more extended scale.

Chemical estimations.

These were carried out by the methods described by Bate-Smith & Bendall (1947*a*). In most cases pH and lactic acid values only were measured, either on the sister muscle to that being used

for elasticity measurements, or on small portions taken from the ends of the muscle actually under measurement. In general, it was found desirable to rely upon pH measurements, which were always very accurate (error $\pm 0{\cdot}03$ pH units) and easily repeatable, rather than upon lactic acid values. The latter vary considerably, even on the same trichloroacetic acid extracts, and reliance cannot be placed upon them unless each value is repeated in triplicate at least, and preferably in quadruplicate. The error of a single determination is $\pm 5\%$. These errors arise mainly in the preliminary oxidation of lactic acid to acetaldehyde. In Fig. 7, in which lactic acid production is plotted against the corresponding fall of pH, the lactic acid values represent averages of at least three replicate estimations, and occasionally as many as five. The figure can therefore be relied upon to be as accurate as possible by the given methods.

Definitions. The pH taken within 5 min. of the death of the animal is referred to as the 'initial pH', and that obtained 2–10 h. after the completion of rigor as the 'ultimate pH'. The 'pH at onset' is the pH at the end of the delay period (see later), measured either directly or by interpolation from a pH/time curve for the particular muscle.

RESULTS

The results are presented in the various figures and tables. In all cases modulus values are given as a percentage of the modulus in full rigor. It should be noted that these final modulus values do not vary in any regular manner with the pre-treatment of the animal nor with its ultimate pH. The average final modulus for thirty-seven experiments was $9{\cdot}2 \times 10^3$ g./cm.^{2} (variance 50%) and average initial modulus $0{\cdot}47 \times 10^3$ (variance 50%).

The types of rigor record

Four main types of rigor record can be distinguished by means of the rigor-recording apparatus described. These are illustrated in Fig. 1 in the form of reproductions of the actual records, and in Fig. 2 by means of modulus/time curves.

In all instances the records exhibit two distinct phases: a 'delay period' during which the modulus of elasticity either does not change at all or increases very slightly, and a phase in which it increases rapidly to its maximum, which may be 10–40 times greater than the initial value. The latter will be referred to as the 'rapid phase'. It is also possible to distinguish a third phase in some cases, which intervenes between the 'delay period' and the 'rapid phase', during which the modulus increases relatively slowly to about 2 or 3 times its initial value. This is followed by the 'rapid phase' proper during which the modulus increases further to 15–40 times its initial value. The duration of these phases, especially of the delay period, shows great variation, but the following types can be distinguished:

(*a*) *Animals with ultimate* pH *values of* 6·1 *or below.* Animals of this type give records such as I and II in Fig. 1. Record I is typical of myanesin-treated animals, the initial pH of the muscle being high (*c.* 7·0). Here, the delay period is well marked, and little or no change in modulus occurs during its course (9 hr.). The rapid phase comes on abruptly with no intermediate phase, and within 1 hr. the modulus has increased to its maximum. Record II is commonly obtained

with untreated animals, which usually struggle violently at death. The initial pH in such cases is much lower than for record I (< 6·60). The 'delay period' is

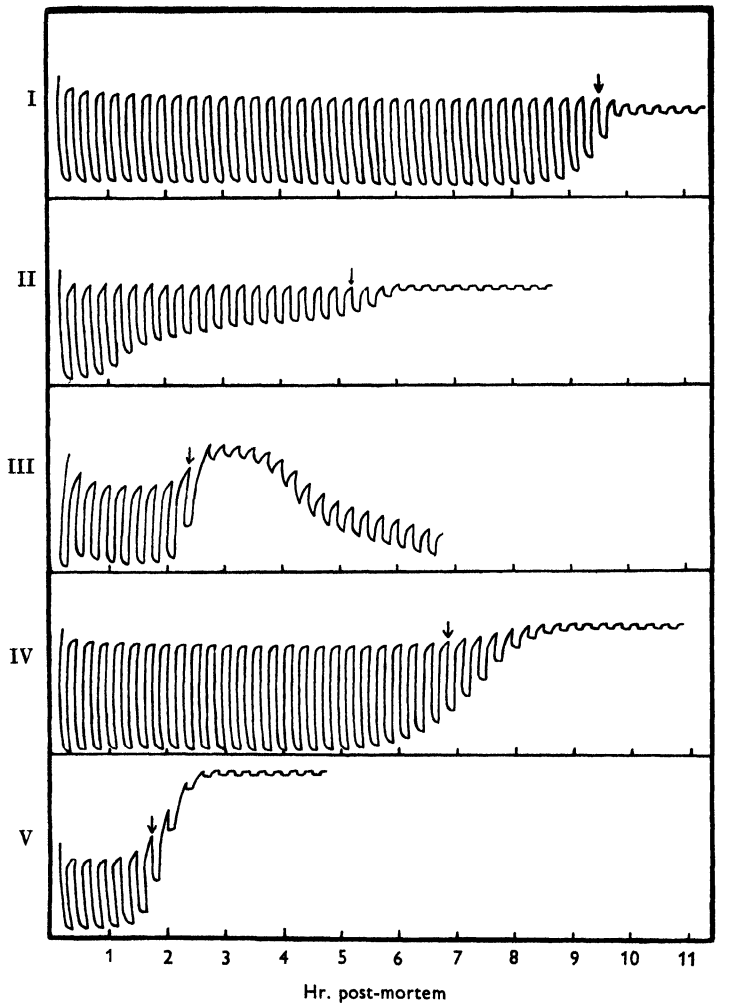

Fig. 1. Types of rigor record.

Type	Initial pH of muscle	Ultimate pH	Temp. (° C.)
I	7·00	6·00	17
II	6·50	5·90	17
III	6·50	5·90	37
IV	7·05	6·50	17
V	7·20	7·20	17

Arrows indicate the end of 'delay period' of rigor.

less well defined, the modulus beginning to change slowly from the moment of death, but there is a well-marked change of rate of increase in modulus when the rapid phase begins. Here again the latter takes about 1 hr. for completion,

which is characteristic of animals with ultimate pH in this range (6·1–5·9). The 'delay period' is, however, much shorter than for record I (5·0 hr.).

No appreciable shortening of the muscles occurs at 17° C. in this type of rigor. Marked shortening (>10%) occurs, however, if the temperature is raised to 37° C. A case of this is illustrated in record III, the muscle of which had initial and ultimate pH values identical with those of record II (pH 6·5 and 5·9 respectively). It should be noted that at 37° C. the shortening is followed by marked lengthening, a phenomenon which has not as yet been fully investigated. In this case, the delay period is reduced to 2 hr., and the rapid phase is completed in about ½ hr. compared with 5 and 1 hr. respectively in the case of record II.

(*b*) *Animals with ultimate* pH *values between* 6·3 *and* 6·7. In order to obtain ultimate pH values in this range the animals must be starved 48–72 hr. They

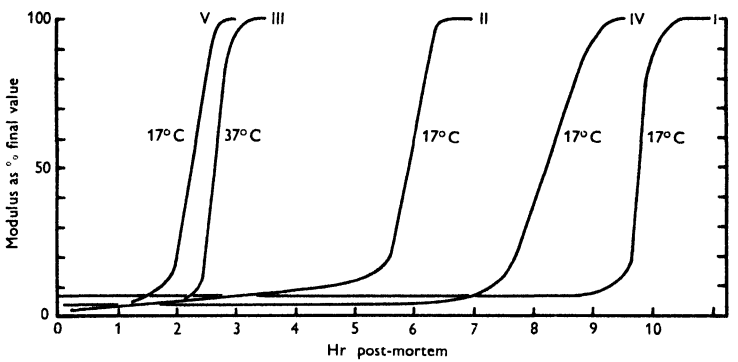

Fig. 2. Modulus curves calculated from rigor records in Fig. 1.

differ from those of type (*a*) in that the delay period is shorter, and less well defined, for any particular initial pH, and the rapid phase more prolonged. Record IV was obtained from such an animal, paralysed with myanesin for ½ hr. before death, and having an initial pH of 7·05 and an ultimate pH of 6·50. If it is compared with record I, in which the initial pH was the same, it will be seen that the delay period for the former lasts 6 hr., against 9 hr. for the latter. This is followed by a phase in which the modulus changes very slowly for 1½ hr., and finally by the rapid phase proper which lasts for about 2 hr. The muscle begins to shorten at the onset of the rapid phase, and at its completion has shortened 5·4%, which is typical in this pH range.

In untreated animals of this type the delay period is further curtailed to 3 hr. or less, and the modulus increases slowly from the moment of death. The initial pH in such cases lies between pH 6·7 and 6·5. The duration of the rapid phase is again about 2 hr., and during its course the muscle shortens 3–5%. The rigor record of such animals is similar to record IV in all respects other than the length of the delay period. Here again, the effect of raising the

temperature to 37° C. is to decrease the length of the delay period and of the rapid phase to less than half and to increase shortening to more than 15%.

(*c*) *Animals, completely exhausted by insulin convulsions, with ultimate* pH *values of* 6·9 *or above.* By giving injections of insulin in doses sufficient to produce violent convulsions it is possible to obtain muscles containing very little lactic acid, and with initial pH between 6·9 and 7·2. In such cases the glycogen reserve is almost completely exhausted, the ultimate pH of the muscle rarely falling below 6·90. This type of rigor is illustrated in record V, and it will be seen that in this particular case the delay period is reduced to 1¼ hr. and is followed immediately by the rapid phase, lasting about 1 hr. The latter is characterized by pronounced shortening (16%). Thus the delay period is of even shorter duration than that of type (*b*), and the rapid phase of about the same duration as that of type (*a*). Two other insulin-treated animals have been studied in the course of these experiments, and in these cases no delay period was apparent, the rapid phase coming on immediately after death. These animals, however, died in convulsions, whereas the animal of record V was killed when death appeared imminent. Two experiments have also been carried out on insulin-treated animals at 37° C. In one case the shortening amounted to 32% and in the other to 45%.

From these results it is apparent that the duration of the delay period is a complex function of the initial and ultimate pH values, whereas the duration of the rapid phase is dependent on the ultimate pH alone. The duration of both phases is decreased markedly by raising the temperature to 37° C. The degree of shortening is also dependent on the ultimate pH and is markedly increased by raising the temperature to 37° C. These various relations will be analysed in detail in the following sections.

Factors affecting the duration of the delay periods

The relation which exists between the duration of the delay period and the initial and ultimate pH of the muscle is demonstrated more fully in Fig. 3, in which the duration is plotted against the ultimate pH for two groups of animals: group 1 with initial pH values of $7{\cdot}0 \pm 0{\cdot}10$ (mainly myanesin treated); group 2 with initial pH values of $6{\cdot}5 \pm 0{\cdot}10$ (the duration is corrected for these small variations in initial pH). It is seen that smooth curves are obtained, the curve for group 1 animals being sigmoid.

If now the curve is analysed in terms of the three types of animals mentioned in the preceding section, animals of type (*a*) (ultimate pH 6·1–5·8) will occur in both groups, those in group 1 having delay periods varying from 7·5 to 10 hr. in duration, and those in group 2 having delay periods from ½ to 5 hr. Animals of type (*b*) (ultimate pH 6·7–6·30), however, can virtually only occur in group 1, and have delay periods varying from 2 to 7 hr. Animals of type (*c*) (ultimate pH 7·0) show little or no delay period, and occur in group 1.

The most striking feature of the group 1 animals is the marked increase in the duration as the ultimate pH falls from 6·7 to 6·4, and the much slower rate of increase as the ultimate pH falls below 6·4, the delay period lasting 5·3 hr. for an ultimate pH of 6·5, but only increasing to 8·9 hr. for an ultimate pH of 6·0. It should also be noted that the slopes of the curves for both groups are very similar below pH 6·35. As the correlation in the two groups between the duration of the delay period and the ultimate pH is so good, it is to be concluded that the rate of change of pH at any value of pH within the range 7·0–5·8 must be remarkably consistent from animal to animal. This conclusion is borne out by the data in Fig. 4, in which the experimentally determined rate of fall of pH ($\Delta pH/\Delta t$) for seventeen animals is plotted against the pH. The

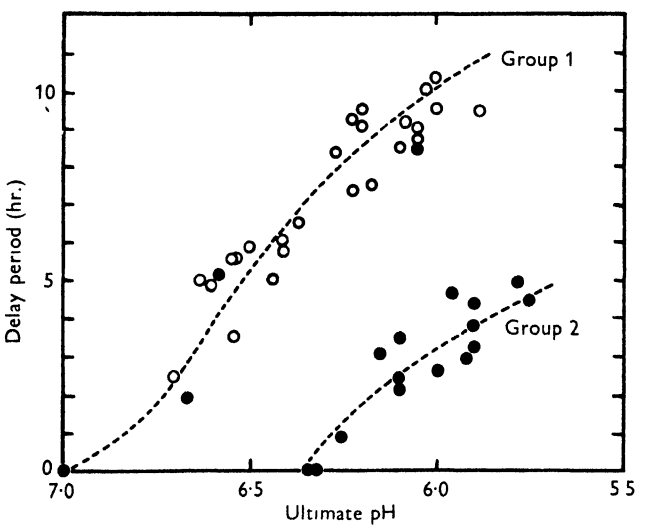

Fig. 3. Relation between duration of 'delay period' and 'ultimate pH' of muscle. Broken lines represent calculated curves. Group 1, 'initial pH' 7·00; Group 2, 'initial pH' 6·50. o, Myanesin-treated animals. •, Untreated animals.

curve is U-shaped, with a minimum at about pH 6·60. This is precisely the region of pH in which the pronounced increase in the delay period occurs.

The curve, integrated over each 0·1 pH unit interval, is shown as a pH/time curve in Fig. 5. It is seen to be S-shaped, with an inflexion in the region 6·55–6·75, and is notably similar in shape to the delay period/ultimate pH curves of Fig. 3. It can be shown, in fact, that the delay period is calculable from a knowledge of the initial and ultimate pH, and the rate of fall of pH over this range. It is possible to do this, because of the linear relation between the pH at onset of rigor and the ultimate pH, illustrated in Fig. 6. It is seen that at pH 7·0 these values are identical, but, at lower ultimate pH values, the difference between them increases until at ultimate pH 5·3 the pH at onset

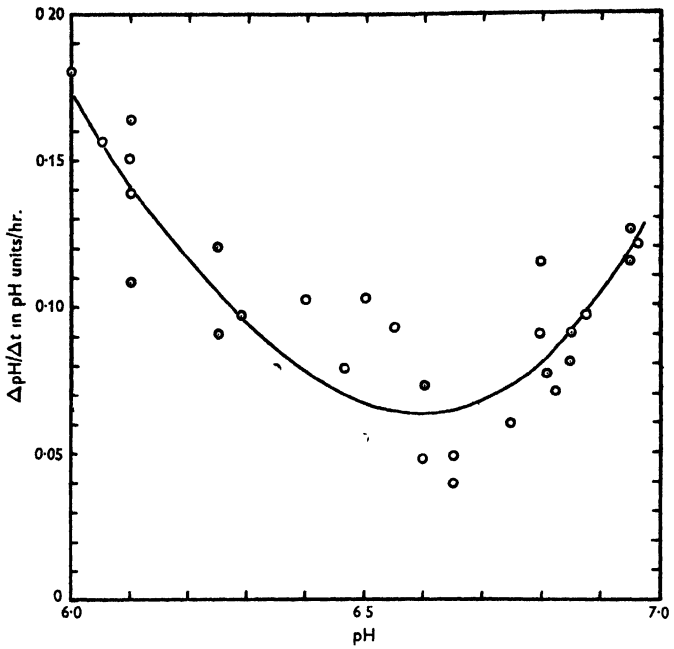

Fig. 4. Rate of glycolysis ($\Delta pH/\Delta t$) at 17° C. plotted against pH of muscle.

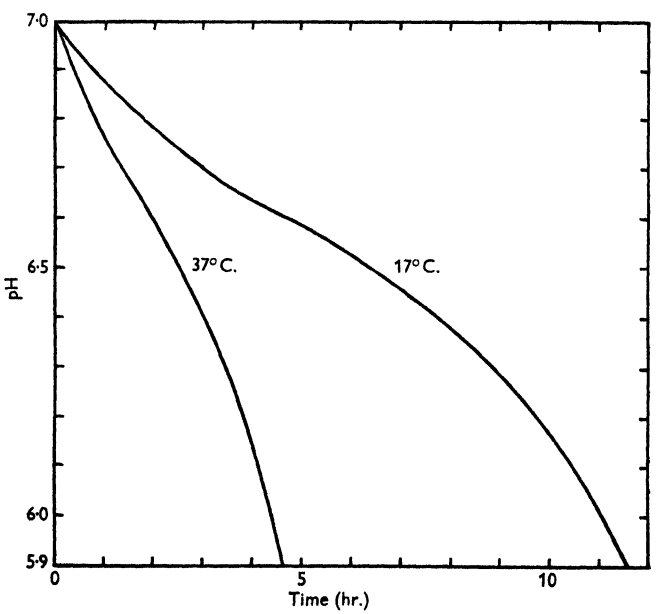

Fig. 5. pH/time curve, calculated by integration of Fig. 4.

is 5·70. Thus, in any calculation of the length of the delay period from the glycolysis curve, this factor must be taken into account. For each value of ultimate pH the pH at onset must first be found from Fig. 6, or from the relation

$$\text{pH at onset} = 0{\cdot}76\ (\text{ultimate pH} + 2{\cdot}2).$$

The time required to reach this value, from an initial pH of either 7·0 or 6·5 (group 1 or 2), is then read off from the glycolysis curve (Fig. 5). This represents the calculated delay period, and is now plotted against the value of the

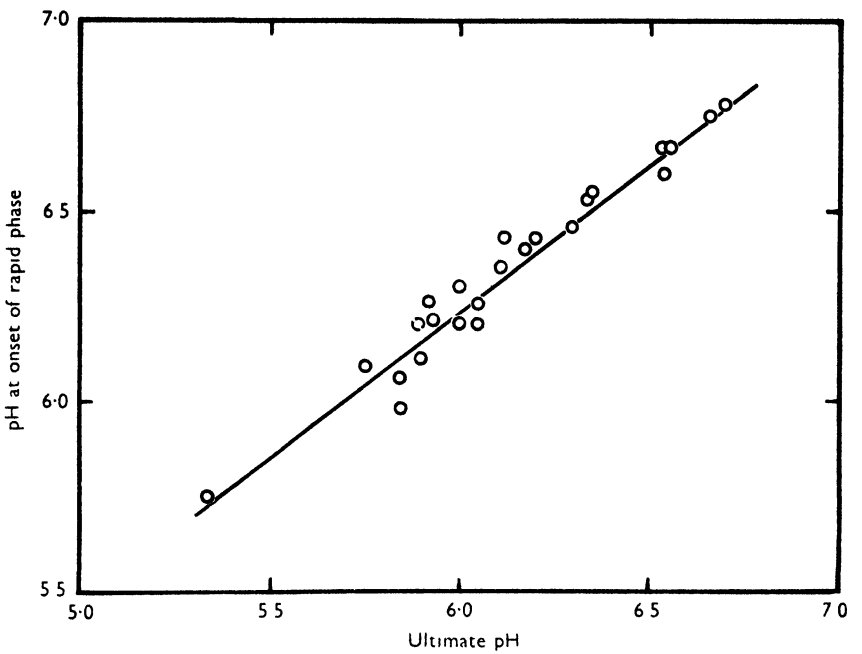

Fig. 6. pH at onset of rapid phase of rigor plotted against ultimate pH.

ultimate pH. The curves, calculated in this way, are shown in Fig. 3 as broken lines. It will be seen that the correlation between the observed points and the calculated curve is good, except for delay periods of 8 hr. or more in group 1. These divergences are mainly due to the difficulty of determining the rate of fall of pH sufficiently accurately in the slow portion of the curve from pH 6·7 to 6·5. The correlation is much better in the group 2 animals, which do not pass through this pH range during the rigor process.

It has been shown, therefore, that the rate of fall of pH is remarkably consistent from animal to animal, and that this explains satisfactorily the observed correlation between the duration of the delay period and the ultimate pH for any given initial pH of the muscle. In its turn the rate of fall of pH is simply related to the rate of production of lactic acid, and thus the duration of

the delay period for any given initial pH is dependent on the amount of lactic acid produced post-mortem, giving curves similar to those in Fig. 3.

The relation between fall of pH and production of lactic acid is shown in Fig. 7. It is seen to be nearly linear and, between the limits pH 7·2–5·8, is given satisfactorily by the equation

$$\frac{\Delta \text{ lactic acid}}{\Delta \text{pH}} = 5{\cdot}70 \text{ mg. lactic acid/g./pH.}$$

This relation is close to that obtained independently from measurements of the buffering capacity of the muscles of twenty animals, so that the pH/time curve from the moment of death is virtually a titration curve of the substance of the muscle with lactic acid.

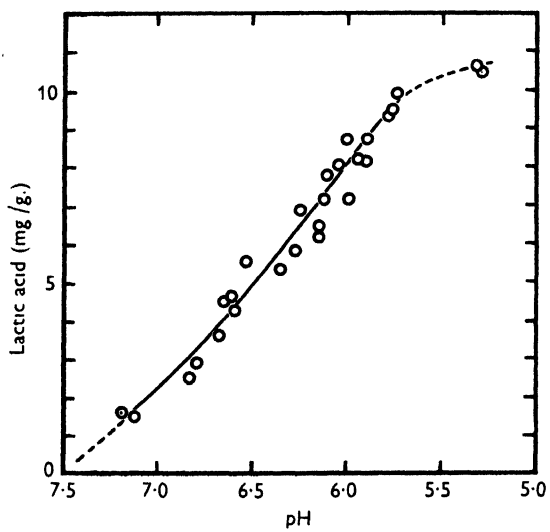

Fig. 7. Lactic acid content of muscle plotted against pH.

There is one implication of this observation that must be clearly stated. The 'internal' pH of living muscle has been calculated by various authors, e.g. Conway & Fearon (1944) to differ considerably from the pH of the extracellular fluid, as it might be determined by indicators, or by a glass electrode applied to the surface of the muscle (Dubuisson, 1939), or thrust into the tissue. There is no evidence from the present work of such marked difference; all the evidence points to the 'internal' pH of resting muscle lying close to 7·0, variation from that value at any given moment being determined by the lactic acid content of the muscle at that moment. Only when the muscles have remained inert for a long period does the lactic acid content approximate to zero and the pH of the muscle increase beyond 7·1.

A second inference is that other reactions taking place post-mortem have little effect on pH by comparison with that due to lactic acid production. So

far as is known these reactions (with the sign of the change in pH they produce) are: loss of CO_2, positive; breakdown of adenosine triphosphate, negative; breakdown of creatine phosphate, positive.

The net result of these changes is a tendency for the pH (in the resting range) to be increased by about 0·3 pH. Thus the pH calculated for muscle after all post-mortem changes have taken place, and after deducting the change due to the lactic acid formed, is, on the average, 7·50 (Bate-Smith, 1948). The effect of these minor changes on the otherwise smooth titration curve of muscle by the lactic acid produced in it takes place for the most part as the pH falls from 7 to 6·8, that is, in the region where there is in any case the greatest uncertainty of measurement. Thereafter the effect is practically pure titration.

Factors determining the initial and ultimate pH *of the muscles*

It has been shown in the previous section that the initial and ultimate pH values of the muscle are critical in determining the time course of rigor, and, therefore, the factors which predetermine these pH values are of the greatest importance.

The main clue to the factors determining the initial pH has been provided by use of myanesin, since in this way it has been possible to study animals paralysed and fully relaxed for as long as 1 hr. before death. The initial pH value characteristic of such animals is 7·0, the maximum variation in fourteen cases being $< \pm 0{\cdot}1$ pH units. On the other hand, untreated animals, which invariably struggle violently during decapitation, have far lower initial pH values, the average for eighteen animals being 6·5, the highest 6·85 (of which there are three cases) and the lowest 6·25. Ten of these animals had pH values in the range 6·45–6·55. These results strongly suggest that the initial pH is determined mainly by the severity of the death struggle. This is borne out by four experiments on bullocks, in which two muscles were chosen for study: the psoas which is involved in the characteristic kicking movement of the hind-limbs when the animal is shot, and the superficial pectoral, involved in movements of the fore-limbs, which remain comparatively quiescent during the death struggle. It was found that in all cases the initial pH of the psoas was close to pH 6·5, whereas that of the superficial pectoral was never below 7·0. Thus all evidence from the present work points to activity immediately before or at death, and this factor alone, being responsible for the variation in initial pH.

The two factors of greatest importance in determining the ultimate pH, appear to be the level of feeding and the degree of fatigue before death. Well-fed and rested rabbits have ultimate pH values lying between 6·2 and 5·3, the average for fifteen animals being 5·90. Only one value (5·3) below 5·75 was recorded. These values seem to be little affected by myanesin treatment, both treated and untreated animals giving about the same average. On the other

hand, animals starved 24–60 hr. have ultimate pH values between 6·3 and 6·7, the average for eleven animals being 6·45. Again myanesin treatment has little effect on the average. The effect of exhausting exercise, brought about in rabbits by means of insulin injected in convulsant doses, is to raise the ultimate pH to the level 6·85–7·25, the average for five animals treated in this way being 7·20. It should be noted that in these cases the initial and ultimate pH values did not differ by more than 0·1 units, and that $< 0·5$ mg. lactic acid/g. was produced post-mortem. It is difficult to subject rabbits to other forms of exercise, but this is quite possible with rats by means of forced running in drums. The results for sixty such experiments with rats are summarized in Table 1, and clearly show that the ultimate pH rises as the exercise becomes more severe. The most severely exercised group have ultimate pH values of the same order as those of insulin-treated rabbits.

TABLE 1. Effect of exercise and starvation on the ultimate pH values of the leg muscles of rats

Treatment	No. in group	Ultimate pH
Well fed, no exercise	17	$5·90 \pm 0·10$
Starved 20 hr., no exercise	15	$6·34 \pm 0·13$
Starved 20 hr., moderate exercise 1½ hr., at 120 ft./min.	13	$6·60 \pm 0·13$
Starved 20 hr., heavy exercise 4–5 hr. at 120 ft./min.	15	$7·05 \pm 0·14$

Thus the most important factor in determining the initial pH of the muscle, i.e. the pH immediately post-mortem, is the severity of the death struggle. The ultimate pH, on the other hand, is little influenced by this factor, but is determined mainly by the level of feeding and the degree of fatigue of the animal immediately before death.

The pH *at the onset of the rapid phase of rigor, and its effect on the rate of increase of modulus*

It has been shown above that the pH at the onset of rigor is determined within narrow limits by the ultimate pH (i.e. by the initial glycogen-reserves of the muscle) the values being identical at pH 7·0, but increasingly divergent for lower ultimate pH values, until with an ultimate pH of 5·30 the pH at onset is 5·70. The relation is governed by the equation

$$\text{pH at onset} = 0·76\ (\text{ultimate pH} + 2·20)$$

The maximum deviation from this relation is about 10% (see Fig. 6).

From this relation it is apparent that the pH at onset for most well-fed animals, with ultimate pH values between 5·80 and 6·10, will lie in the range 6·10–6·30. This accounts for the regularity with which in previous work (Bate-Smith, 1939; Bate-Smith & Bendall, 1947) the onset of rigor in well-fed animals was associated with a pH in the neighbourhood of 6·3. Clearly, exceptionally well-fed animals with ultimate pH values below 5·75 would have provided

exceptions to this rule, since the pH at onset would have been 6·05 or below. Such animals have, however, occurred so infrequently in these investigations that the original generalization was justified on the evidence available at the time.

The effect of pH at onset on the rate of increase of modulus in the rapid phase has been demonstrated in a limited way in Figs. 1 and 2. In Fig. 8 the effect is shown in more detail for twenty-seven experiments. The rate is given

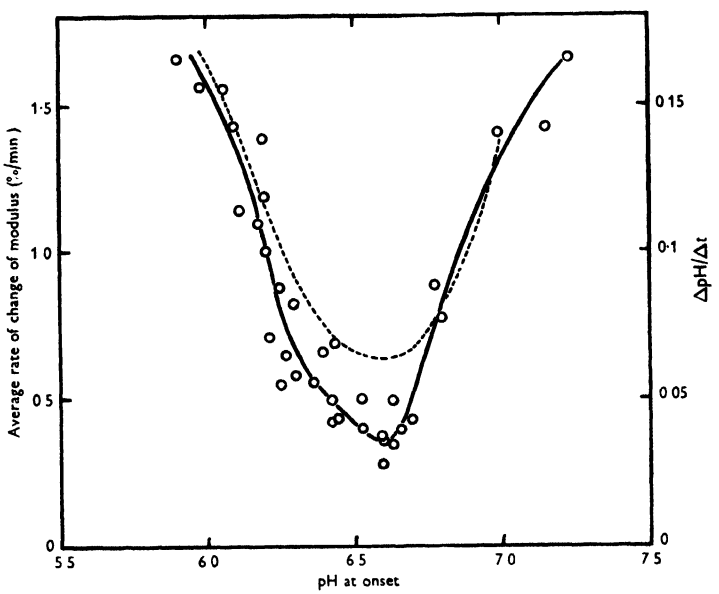

Fig. 8. Average rate of change of modulus from onset of rigor to 50% stage plotted against pH at onset (continuous line). The curve relating rate of change of pH ($\Delta pH/\Delta t$) to pH is superimposed for reference (broken line).

as the average rate from the beginning of the rapid phase to the point of 50% change of modulus. It will be seen that the curve relating these two variables is markedly U-shaped, with a trough extending from pH 6·4 to 6·7 in which the rate is at a minimum, a rapidly ascending limb as the pH falls below 6·4 with a maximum at least four times greater than the minimum, and another rapidly ascending limb as the pH rises above pH 6·7. The shape of the curve immediately recalls that of the ($\Delta pH/\Delta t$)/pH curve (Fig. 4), superimposed for comparison, the trough of which occurs fairly sharply at pH 6·60. The similarity of the two curves is too marked to be merely coincidental, and can be explained in terms of breakdown of A.T.P., as will be demonstrated in the discussion.

The effect of temperature on the time course of rigor.

The effect of temperature on the duration of the delay period and of the rapid phase, and on the degree of shortening, has been mentioned briefly. By raising the temperature from 17 to 37° C. the delay period is shortened 2½ times. This effect is illustrated in Table 2 for paired muscles, and is seen to be remarkably consistent from animal to animal. Between 17 and 37° C. the Q_{10} of the process is thus about 1·60.

TABLE 2. Effect of temperature on the duration of the delay period

Initial pH	Final pH	Delay 17° C.	Delay 37° C.	Ratio of delay at 17 to delay at 37° C.	Q_{10}
7·05	6·42	6·5	2·66	2·44	1·560
7·05	5·80	10·0	3·8	2·63	1·620
6·75	6·58	2·67	1·10	2·42	1·555
6·60	6·32	c. 1·0	c. 0·3	—	—
6·52	5·76	5·2	2·15	2·42	1·555
6·32	5·32	3·66	1·33	2·75	1·660
			Average	2·53	1·59

Below 17° C. the Q_{10} decreases. Thus in an experiment in which one psoas muscle was placed at 37° C. and its sister muscle at 3·5° C., the delay period lasted 3·8 hr. at 37° C. and 13·0 hr. at 3·5° C. (Both muscles had an initial pH of 7·05 and a final pH of 5·80.)

Thus
$$\frac{\text{Delay } 3{\cdot}5^\circ \text{ C.}}{\text{Delay } 37^\circ \text{ C.}} = 3{\cdot}4,$$

which gives a Q_{10} of 1·44. Again comparing the delay at 3·5° C. with the delay for a sample (no. 2 in Table 2) at 17° C. having the nearest initial and ultimate pH:

$$\frac{\text{Delay } 3{\cdot}5^\circ \text{ C.}}{\text{Delay } 17^\circ \text{ C.}} = 1{\cdot}3.$$

This gives a Q_{10} of 1·22 for the range 3·5–17° C. With a Q_{10} of this low order it is clear that little or no difference would be detected between similar samples at 0 and 17° C. respectively unless the delay period exceeded 4 hr., since the scatter is high for such short delay periods (see Fig. 3, group 2). This accounts for the fact that only insignificant differences in duration between samples at 0 and 25° C. were found by Bate-Smith (1939).

The effect of temperature on the duration of the rapid phase is shown in Table 3. Here the time for 50% change of modulus is compared at the two temperatures. It is seen that there is considerable variation in the ratio $t_{17^\circ\text{C.}}/t_{37^\circ\text{C.}}$ due mainly to the difficulty of measuring the time for half-change accurately at the very high rates at 37° C. In some cases the half-change occurs within one cycle of loading and unloading of the muscle (16 min.). The values would give an approximate Q_{10} of 1·45 for the process. The ratio for two

similar samples at 3·5 and 37° C. was ($t_{3\cdot5}/t_{37°\,C.}$) = 5·3. This gives a Q_{10} of 1·64 for this range. Similarly $t_{3\cdot5}/t_{17°\,C.}$ for similar samples was 2·05, giving a Q_{10} of about 1·7.

Both temperature and pH have a profound effect on shortening, as shown in Table 4. The temperature coefficient is evidently considerably higher than the Q_{10} for the duration of the delay period. At 37° C. and pH values below 6·45, lengthening of the muscle (up to 25 %) frequently occurs after the shortening has reached a maximum (see record III, Fig. 1). A more detailed account of the phenomenon of shortening and lengthening in rigor will be given elsewhere.

TABLE 3

pH at onset	Time (t) for change of modulus from 0 (min.) to 50 %			
	17° C.	37° C.	Ratio t_{17} to $t_{37°\,C.}$	Q_{10}
6·63	95	36	2·64	1·62
6·60	182	100	1·82	1·35
6·10	35	16	2·20	1·48
6·05	44	25	1·75	1·33
6·05	42	19	2·20	1·48
5·75	55	26	2·10	1·45
			Average 2·14	1·45

TABLE 4. Shortening of muscles under an intermittent load of 50 g./cm.²

pH	Shortening (% initial length)	
	17° C.	37° C.
6·0	0·0 ± 0·5	11·0 ± 2·0
6·2	1·5 ± 0·5	15·5 ± 2·0
6·4	2·5 ± 1·0	18·5 ± 2·0
6·6	4·4 ± 1·0	21·5 ± 3·0
6·8	7·0 ± 1·5	25·0 ± 3·0
7·0	9·0 ± 1·5	30·0 ± 3·0
7·2	12·5 ± 1·0	36·0 ± 5·0

DISCUSSION

The role of adenosinetriphosphate in the rigor process

In the earlier paper (Bate-Smith & Bendall, 1947*a*) it was shown that the event most closely related in time to the onset of rigor was the disappearance of ATP from the muscles, which occurs at or slightly before the onset of the rapid phase, the ATP level remaining high during the delay period proper. This relation was shown to hold irrespective of the pH at onset of rigor.

Analysis of the four experiments detailed in the earlier paper, together with eight further exactly similar experiments, have confirmed these findings, and have shown that the disappearance of ATP is related almost linearly to the decrease in extensibility of the muscle, and inversely to the change in modulus.

The extensibility begins to change rapidly as soon as the ATP level falls below 85 % of its initial value of 0·435 ± 0·055 mg. ATP-P/g., and the change is virtually completed when the ATP level has fallen below 15 % of this value.

The hypothesis put forward in 1947 to explain this phenomenon was that the level of ATP in the muscle represents a balance between breakdown of ATP and its resynthesis from the anaerobic glycolytic cycle (Needham, 1942; Lipmann, 1941), but a satisfactory explanation of the mechanism of ATP breakdown itself was not put forward at the time. It is now possible, however, to take the argument further. Kalckar (1944) has shown, by means of radio-phosphorus, that the rate of turnover of ATP-P in resting, living rabbit muscle is very high (20–30 μg. ATP-P/min./g.), and is of the same order as that of phosphocreatine. Now it is not likely that the turnover rate in the muscle post-mortem would differ much from this value at pH 7·0 and 37° C. If the Embden-Meyerhof scheme of glycolysis (Needham, 1942) holds true, in which ATP-P breakdown is taken to be the primary reaction determining the rate of glycolysis, it should be possible to calculate the post-mortem rate of turnover of ATP, under conditions in which the initial ATP-P level is being maintained, from the observed rate of glycolysis at pH 7·0 (Fig. 4). Calculated in this way, by assuming that production of 1 mol. of lactic acid gives rise to $1\frac{1}{2}$–2 mol. ATP-P (Lipmann, 1941) the rate at 17° C. would lie in the range 7·0–9·3 μg./min./g. As the Q_{10} of glycolysis in this temperature range has been shown to be 1·60, it follows that the rate at 37° C. would lie in the range 17·8–23·7 μg. ATP-P/min./g. This is reasonably close to Kalckar's value, and proves that the rate of turnover of ATP in surviving muscle is very similar to the resting rate in the living muscle at pH 7·0, and that the ATP level at any time represents not a static, but a dynamic equilibrium between breakdown and synthesis.

The glycolysis results also give a useful clue to the nature of the enzymes responsible for the ATP-turnover. From the foregoing argument it is apparent that the rate of glycolysis should be simply dependent on the rate of ATP-P turnover, and thus the latter should be found to vary with pH in a manner similar to the rate of glycolysis/pH curve (Fig. 4), having a minimum at pH 6·60, and two sharply rising limbs on the acid and alkaline sides of this point. This curve closely resembles the activity/pH curve for myosin obtained by Engelhardt (1942), the latter also being U-shaped, but with a minimum at pH 6·80. Unfortunately, there are no points on Engelhardt's curve at pH 6·6–6·7, and it is possible that the true minimum of the activity was not obtained in his experiments. It would, therefore, appear probable that myosin is the enzyme responsible for ATP-turnover, and it becomes unnecessary to postulate, as was done in the earlier paper (1947), the participation of Sakov's unspecific polyphosphatase. Moreover, the latter has a completely different activity/pH curve, having a maximum at about pH 6·0, and a minimum at pH 7·0.

Myosin cannot, however, be the only enzyme responsible, since it catalyses the reaction ATP $\rightarrow$ ADP + P, and would give rise to large amounts of ADP in the muscle post-mortem. Bailey (1948) has shown that ADP never accumulates

in appreciable amounts in rabbit muscle post-mortem. However, the intervention of Kalckar's myokinase (1934), or a similar enzyme catalysing the reaction 2 ADP → ATP + Adenylic acid is the only necessary additional consideration, provided that the activity of ATP-ase is the rate-determining factor.

The turnover of ATP, viewed in this way, provides a satisfactory explanation of the events leading to rigor mortis. The delay period shown by types *a* and *b* (pp. 49, 50 and 51) would be a phase in which the system of breakdown (myosin and myokinase) is in balance with the system of resynthesis of ATP (the glycolytic cycle). As has been shown, the duration of this period is determined solely by the maximum fall of pH post-mortem, or in other terms by the extent of lactic acid production. Thus, providing that the back pressure of ATP resynthesis from the glycolytic cycle is sufficiently high the muscle will not pass into rigor. As soon, however, as the muscle is exhausted, or seriously depleted, of glycogen and the production of lactic acid begins to flag, ATP breaks down in excess of its resynthesis, the extensibility of the muscle decreases, and the rapid phase of onset of rigor begins.

The duration of the rapid phase will be determined by the relative rates of resynthesis and breakdown of ATP. At pH values at which ATP turnover is slow, it would be expected that breakdown would overtake the flagging resynthesis slowly, a considerable time being necessary for the ATP level to fall from 85 to 20 % of its initial value. On the other hand, at fast rates of turnover, breakdown would overtake flagging resynthesis in a shorter time. This deduction is borne out by the relation between the rate of glycolysis and rate of change of modulus at varying pH shown in Fig. 8, the two rates following one another closely.

There is, however, another complicating factor in that the pH at the onset of rigor only coincides with the ultimate pH at pH 7·0, but differs from it increasingly at lower ultimate pH values, until at an ultimate pH of 5·3 the pH at onset is 5·70. In fact, an ultimate pH of 5·3 appears to be a limiting value, beyond which glycolysis is completely inhibited, since in many species of animal, considerable quantities of glycogen are frequently found at this ultimate pH, although the muscle is in full rigor and glycolysis is at a standstill. This suggests that although glycolysis can still take place at high rates at the lower pH values it cannot keep going at a rate sufficient to balance breakdown of ATP.

There are several possible explanations of this phenomenon, amongst which the most probable is that one or other of the enzymes involved in glycolysis is increasingly inhibited as the pH falls. Sakov (1941), for example, has shown that the enzyme responsible for the reaction ATP + Hexose-6-P → ADP + Hexose-di-P has its maximum activity at pH 7·0, and is almost completely inhibited below pH 5·5. This is in line with the observation by Needham (1942)

and others, that hexose-6-P often accumulates in muscle extracts, and would also explain the occurrence of large amounts of esterified phosphate (non-labile) in rigor muscle at pH values below 6·30 (Bate-Smith & Bendall 1947*a*).

Thus, it is concluded that ATP is necessary to prevent the muscle passing into rigor; that the ATP of the muscle post-mortem has a high rate of turnover; that during the delay period the breakdown of ATP (by myosin and myokinase) is balanced by its resynthesis from the glycolytic cycle; that the rapid phase of rigor begins when this balance ceases to be maintained as a result of insufficiency of glycogen, and proceeds at a rate determined by the rate of turnover of ATP, which is itself dependent on pH.

SUMMARY

1. In rabbits, three types of rigor are distinguishable by means of the records of continuous changes in modulus: type *a*, well-fed animals with high glycogen-reserves giving a long delay before onset of rigor; type *b*, starved animals with lowered reserves, giving a shorter delay and type *c*, exhausted animals with little or no reserve, giving a very short delay or none at all.

2. It is shown that the duration of the delay period varies with the ultimate pH, if animals of the same initial pH are compared, and that this relation obtains because the rate of fall of pH in any pH range is remarkably consistent from animal to animal.

3. The initial pH of the muscle is shown to be mainly dependent on the severity of the death struggle, whereas the ultimate pH is determined by the level of feeding and the degree of fatigue of the animal immediately before death.

4. The pH at the onset of rigor is linearly related to the ultimate pH, being identical with the latter at pH 7·0, but diverging from it at lower pH values until at ultimate pH 5·3 the pH at onset is 5·7.

5. The pH at onset determines the rate of change of modulus in the rapid phase, these two values being related by a U-shaped curve, with a marked minimum between pH 6·4 and 6·7. This minimum coincides with the minimum in the $(\Delta pH/\Delta t)/pH$ curve.

6. The effect of temperature on the time course of rigor is to decrease the duration of the delay period and the rapid phase: the former is decreased 2·5 times as the temperature is raised from 17 to 37° C., giving a Q_{10} of 1·60. The Q_{10} in the range 3–17° C. appears to be lower (1·22). The Q_{10} of the rate of change of modulus in the rapid phase is also close to 1·60. The effect of raising the temperature from 17 to 37° C. on shortening of the muscle during rigor is to increase it 6–10 times at pH values below 6·5, and about 3 times at pH 6·5 to 6·8.

7. It is confirmed that the rapid disappearance of ATP from the muscle coincides with the change in extension in the rapid phase of rigor. It is deduced

that the rate of turnover of ATP determines the rate of glycolysis; that during the delay period the breakdown of ATP is exactly balanced by its resynthesis from the glycolytic cycle; and that the rapid phase of rigor occurs when the reserve of glycogen in the muscle is almost exhausted, resynthesis then being unable to keep pace with breakdown.

8. The rate of ATP turnover can be deduced from the rate of glycolysis under conditions in which resynthesis and breakdown of ATP exactly balance one another. This rate, calculated at pH 7·0 and 37° C. for surviving muscle, is close to the rate of turnover in intact, living rabbit muscle, obtained by the use of radio-phosphorus (Kalckar, 1944). The $(\Delta pH/\Delta t)/pH$ curve can, therefore, be re-interpreted as a $(\Delta ATP/\Delta t)/pH$ curve, and in this form resembles closely the pH/activity curve of myosin. It is deduced that myosin, together with myokinase, are the enzymes responsible for ATP-turnover in the muscle post-mortem. Sakov's unspecific polyphosphatase is thus no longer needed to explain the results satisfactorily.

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THE DECREASE IN FREQUENCY OF CONTRACTION OF THE JEJUNUM AFTER TRANSPLANTATION TO THE ILEUM

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The observation that the rhythmic contractions of the jejunum of the dog occur at a higher rate than those in the ileum was made eighty years ago by Legros & Onimus (1869). It was not until forty-six years later that Alvarez (1915) emphasized that the rate of rhythmic contraction of any segment of the small intestine of the rabbit varies inversely with its distance from the pylorus. He also noted that, in excised segments of rabbits' intestine contracting in a saline bath, the differences in rate persist, but at a frequency some 25% slower than in the intact animal.

The findings of Alvarez in the rabbit were confirmed in the dog by Castleton (1934), and by Douglas & Mann (1939), who found that the rate of rhythmic contraction is constant for any given segment of intestine over long periods, and is unaffected by feeding, fasting or sleep or by section of the extrinsic nerves. The rate of contraction of the duodenum of the dog is similar to that of the jejunum and as constant (Douglas, 1948). The small intestine of dogs appears to respond to increased work, not by an increase in the rate of contraction, but by increase in the amplitude and vigour of the contractions.

In the course of another investigation it was necessary to transplant a segment of jejunum of the dog to the lower ileum, and to study the rates of rhythmic contraction before and after transplantation. The pre-operative rate had been between seventeen and eighteen contractions per min.; after transplantation it fell to about twelve contractions per min.—a rate similar to that found in the ileum. This result was so unexpected that it was decided to investigate the cause of the fall in rate.

METHODS

Two lines of investigation were followed: (1) long-term observations were made in surviving animals with exteriorized loops of jejunum, and (2) acute experiments were carried out in anaesthetized animals with the intestine exposed in a saline bath. Dogs were used throughout.

Survival experiments

In ten dogs, a loop of jejunum 50 cm. distant from the pylorus was exteriorized and enclosed in a bi-pedicled tube of skin according to the method described originally by Biebl (1930) (Fig. 1).

The following points were found to be important in the surgical technique:

(*a*) Bitches are more suitable than dogs because of their lax abdominal skin and the absence of the penis.

(*b*) The skin incisions should be at least 5 cm. apart to provide a tube of skin wide enough to cover the loop without tension.

(*c*) Mobilization of the abdominal skin down to the flanks is essential to allow the abdominal incision to be closed easily.

(*d*) In addition to the skin sutures, interrupted sutures of fine silk (0·17 mm. in diameter) should be inserted at intervals of 0·5 cm. in the stout subcutaneous layer, to prevent dehiscence of the skin round the exteriorized loop of intestine and the consequent risk of an intestinal fistula.

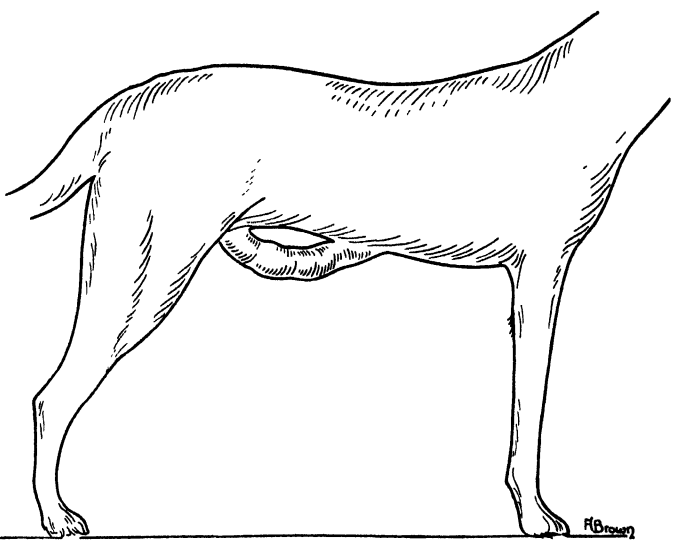

Fig. 1. Diagram of bitch with exteriorized loop of jejunum enclosed in bi-pedicled tube of skin.

Plan of the survival experiments. After recovery from the exteriorization operation, the animals were trained to lie on a padded observation table, and the activity of the intestine was recorded from day to day by an air displacement system (Douglas & Mann, 1941). Throughout this preliminary observation period of 3–5 weeks, the mean rate per minute of the rhythmic contractions was established for each loop.

Each animal was then submitted to a second operation designed to elucidate the effect of various types of section of the intestine on the contraction rate of the exteriorized loop. After recovery from this operation, the day to day observations on the activity of the loop were continued for a period of 5–12 months. The various operations were as follows (Fig. 2):

(*a*) Transplantation of the jejunal loop to the lower ileum—two animals (Fig. 2*B*).

(*b*) Isolation of the loop, both ends being brought out through stab wounds in the flank as in the preparation of a Thiry-Vella fistula—two animals (Fig. 2*C*).

(*c*) Section and re-anastomosis of the intestine 15 cm. proximal to the loop—three animals (Fig. 2*D*).

(*d*) Section and re-anastomosis of the intestine 15 cm. distal to the loop—two animals (Fig. 2*E*).

(*e*) Hemi-section and resuture of the intestine 15 cm. proximal to the loop—one animal (Fig. 2*F*).

Acute experiments

Nineteen dogs were used in the acute experiments. They were anaesthetized with pento-barbitone in doses of 25 mg./kg. intravenously. An endotracheal catheter was then passed and a light plane of anaesthesia maintained with ether. Morphine sulphate administered in 4 mg./kg. doses intravenously was found to promote active contractions of the intestine.

The spinal cord was exposed by laminectomy in the upper thoracic region and divided at about the second thoracic segment. A malleable wire was then passed down the theca to the sacral region, vigorously rotated, and withdrawn, destroying the cord below the level of section; the central connexions of the sympathetic supply to the intestine were thereby destroyed. At this point it was found expedient to institute controlled respiration with a positive insufflation pump (stroke volume 150 c.c., rate 25/min.). The abdomen was opened by a midline incision; one balloon was placed in the descending part of the duodenum and another in the upper jejunum at a measured distance

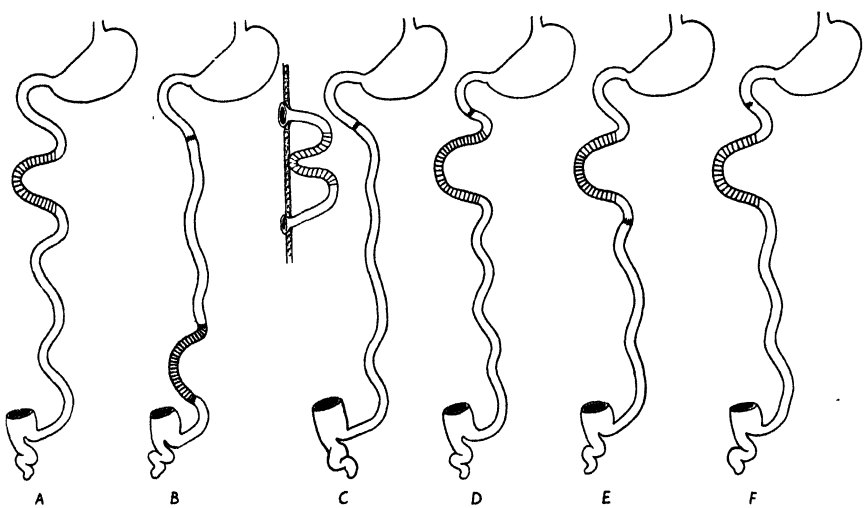

Fig. 2. Diagram of the various operations performed on the jejunum. *A*, the exteriorized loop; *B*, loop transplanted to lower ileum; *C*, loop isolated; *D*, section and re-anastomosis proximal to the loop; *E*, section and re-anastomosis distal to the loop; *F*, hemi-section and resuture proximal to the loop.

from the pylorus. In five of the experiments, the vagi were severed as they lay on the abdominal part of the oesophagus.

The whole animal was then placed in a constant temperature saline bath maintained at 37–38° C. The balloons were connected to water manometers and inflated to a pressure of 20 cm. of water; the movements of the water in the manometers were recorded by a kymograph.

After a control tracing lasting 20–30 min., one of the following manipulations was performed on the bowel midway between the balloons:

(*a*) An intestinal clamp was applied crushing the gut.

(*b*) The wall of the bowel was infiltrated with ½% procaine hydrochloride.

(*c*) The bowel was completely severed.

After these interventions the tracing was continued for 1–2 hr.

In order to demonstrate the effect of these manoeuvres on the rate of the rhythmic contractions it was essential that there should be active and regular contractions in both duodenum and jejunum simultaneously. This proved to be the chief difficulty of the method, since, although the duodenum was usually active, the jejunum was often quiescent. In overcoming this difficulty it was found of value to allow 2–3 hr. to elapse between destruction of the cord and the making of the

intestinal tracing, and to use a shallow plane of ether anaesthesia, relying on the morphine sulphate for supplementary sedation.

At the end of the experiment the dogs were painlessly killed while under the anaesthetic. The tracing was then fixed, ruled off in intervals of 1 min. and the number of rhythmic contractions per minute counted.

RESULTS

Survival experiments (Table 1)

Control period. In the ten dogs with exteriorized jejunal loops, in the control period, the contraction rate was relatively constant for each animal. There was also little variation from dog to dog, the slowest mean rate being 17·1 contractions per min. and the highest 18·2.

TABLE 1. The effect of various operations on the rate of contraction of segments of exteriorized jejunum in surviving dogs. The figure following the mean is the standard error of the mean and the figure in brackets the number of observations upon which the mean is based

Dog	Mean rate of contractions/min. Control	Operation	Mean rate of contractions/min. Post-operative	Mean difference
1	17·6 ± 0·12 (46)	Transplantation to ileum	12·01 ± 0·05 (125)	−5·6
2	17·1 ± 0·13 (51)	Transplantation to ileum	15·03 ± 0·05 (168)	−2·07
3	17·5 ± 0·08 (53)	Isolation	12·03 ± 0·08 (84)	−5·02
4	17·8 ± 0·09 (76)	Isolation	14·7 ± 0·08 (112)	−3·1
5	17·7 ± 0·07 (72)	Proximal section	14·3 ± 0·05 (229)	−3·4
6	18·3 ± 0·08 (88)	Proximal section	14·1 ± 0·04 (259)	−4·2
7	18·1 ± 0·13 (56)	Proximal section	12·7 ± 0·07 (97)	−5·4
8	18·0 ± 0·07 (112)	Distal section	17·8 ± 0·06 (92)	−0·2
9	17·4 ± 0·08 (83)	Distal section	17·6 ± 0·05 (131)	+0·2
10	18·1 ± 0·08 (75)	Proximal hemi-section	18·2 ± 0·04 (288)	+0·1

Effect of transplantation. In both dogs transplantation of the loop to the lower ileum resulted in a fall in the mean contraction rate, in the first by 5·6 contractions per min. (28·6 %) and in the second by 2·07 (12·2 %). The animals were observed for more than a year and there was no return to the original rate.

Effect of isolation. In both animals in which the loop was isolated with the nerve and blood supply intact, a similar fall in rate occurred, of 5·02 and 3·1 contractions per min. respectively (28 and 17·4 %).

Effect of proximal section. The operation of section and re-anastomosis of the intestine 15 cm. proximal to the exteriorized loop resulted in a fall in the contraction rate of the loop in all three animals by 3·4, 4·2 and 5·4 contractions per min. (19·2, 23 and 27 %) respectively (Figs. 3 and 4). Recovery in rate has not taken place 9 months after operation.

Effect of distal section. In contrast to the effect of the above operations, section and re-anastomosis of the intestine 15 cm. distal to the loop was not followed by a change in contraction rate in either of the two animals in which the operation was carried out (Fig. 5).

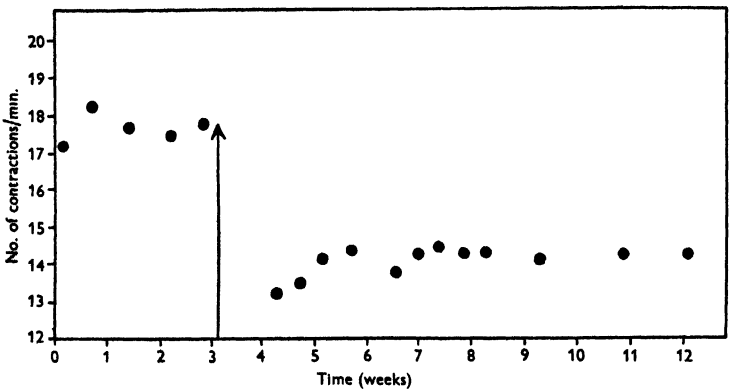

Fig. 3. Mean rate of contraction of jejunal loop. Ordinates: mean rate of contractions per min. Abscissae: time in weeks from original observation. At arrow, the operation of proximal section and re-anastomosis. The fall in mean rate should be noted.

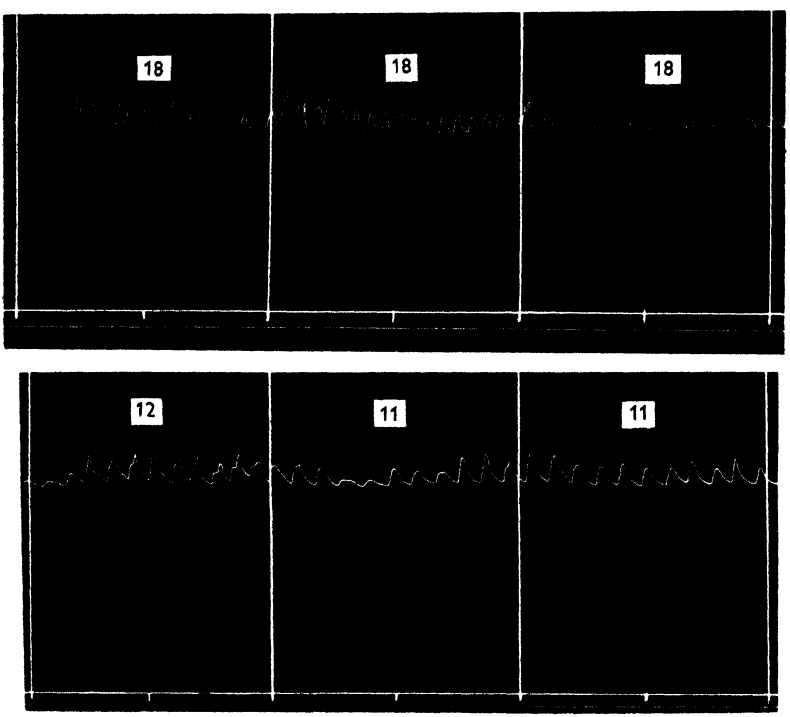

Fig. 4 Contractions of exteriorized jejunal loop. Upper tracing, control period. Lower tracing, after proximal section and re-anastomosis. Time in minutes. The fall in rate can be seen, and the frequency is shown above each record.

Effect of proximal hemi-section. In order to observe the effect of an operation of comparable severity on the intestine proximal to the loop, which did not involve complete interruption of continuity, the antimesenteric half of the circumference of the intestine was divided and resutured in one animal. This operation was not followed by a change in contraction rate.

Acute experiments

Application of clamp. In seven experiments in this group a clamp was applied between duodenum and jejunum (Table 2). A fall in the frequency of rhythmic contractions in the jejunum occurred in all the experiments, while the frequency of the duodenal contractions remained unaltered. The onset of the slowing was usually immediate. The mean fall in the seven experiments was 2·9 beats per min.

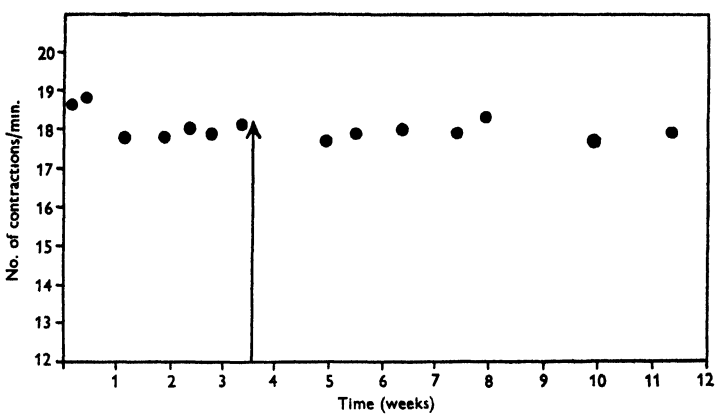

Fig. 5. Mean rate of contraction of jejunal loop. Ordinates: mean rate of contraction per min. Abscissae: time in weeks from original observation. At arrow, the operation of distal section and re-anastomosis. The mean rate is unaltered.

After removal of the clamp, recovery of the rate of contraction to the original level occurred in three of the experiments. In the remaining four, the jejunal contractions did not regain their original rate.

Injection of procaine hydrochloride. In ten experiments the wall of the intestine, midway between the two balloons, was infiltrated in a 'ring' fashion with 10 c.c. of ½% solution of procaine hydrochloride. Slowing of the jejunal rate was noted in each experiment in this group, the mean fall being 3·0 beats per min. (Figs. 6 and 7, and Table 3).

DISCUSSION

Reference to an investigation on the effect of transplantation of segments of intestine on the contraction rate has not been found in the literature, though Castleton (1934) isolated segments of jejunum and divided their mesentery. He found that this operation caused a fall in rate of about 25%.

TABLE 2. Effect of application of a clamp between duodenum and jejunum on the rate of contractions. Upper balloon in duodenum, lower balloon as noted in column 1: acute experiments in dogs. Statistical presentation as in Table 1

Distance of lower balloon from pylorus (cm.)	Mean rate of contractions/min.				Difference between means	
	Before clamping		After clamping			
	Duodenum	Jejunum	Duodenum	Jejunum	Duodenum	Jejunum
30	18·2±0·20 (9)	17·8±0·20 (9)	17·7±0·15 (20)	13·6±0·11 (21)	−0·5	−4·2
37	16·5±0·17 (17)	16·3±0·17 (17)	16·7±0·12 (24)	14·2±0·11 (20)	+0·2	−2·1
45	16·3±0·11 (35)	16·0±0·12 (24)	16·7±0·09 (29)	14·6±0·14 (26)	+0·4	−1·4
50	16·1±0·26 (9)	15·8±0·14 (12)	16·6±0·18 (19)	12·9±0·14 (18)	+0·5	−2·9
50	17·0±0·11 (21)	16·8±0·10 (16)	16·7±0·11 (12)	13·3±0·21 (10)	−0·3	−3·5
50	17·2±0·20 (15)	16·8±0·10 (15)	17·6±0·20 (21)	12·8±0·13 (20)	+0·4	−4·0
50	17·7±0·20 (11)	16·5±0·14 (15)	17·4±0·13 (28)	14·4±0·12 (28)	−0·3	−2·1

TABLE 3. Effect of division or of infiltration with ½% procaine hydrochloride of the wall of the intestine between duodenum and jejunum on the rate of contractions. Upper balloon in duodenum, lower balloon as in column 1: acute experiments in dogs. Statistical presentation as in Table 1

Distance of lower balloon from pylorus (cm.)	Intervention	Mean rate of contractions/min.				Difference between means	
		Before intervention		After intervention			
		Duodenum	Jejunum	Duodenum	Jejunum	Duodenum	Jejunum
45	Division	19·2±0·18 (10)	16·3±0·18 (10)	19·2±0·14 (34)	13·3±0·08 (30)	0	−3·0
50	Division	18·5±0·11 (12)	18·3±0·20 (12)	17·0±0·09 (22)	12·6±0·11 (39)	−1·5	−5·7
37*	Infiltration	14·8±0·16 (26)	14·8±0·14 (25)	15·4±0·14 (21)	12·7±0·14 (24)	+0·6	−2·1
37	Infiltration	17·3±0·15 (11)	17·3±0·12 (11)	17·3±0·09 (45)	13·9±0·11 (39)	0	−3·4
45*	Infiltration	15·5±0·11 (20)	15·4±0·14 (18)	15·7±0·20 (9)	12·6±0·15 (15)	+0·2	−2·8
48	Infiltration	17·0±0·09 (21)	16·7±0·10 (17)	16·5±0·11 (20)	13·2±0·08 (24)	−0·5	−3·5
50	Infiltration	19·1±0·11 (18)	17·2±0·12 (19)	19·0±0·14 (11)	13·1±0·15 (15)	−0·1	−4·1
50*	Infiltration	16·5±0·16 (27)	16·5±0·14 (26)	16·3±0·16 (23)	13·1±0·13 (21)	−0·2	−3·4
50*	Infiltration	14·7±0·07 (31)	14·3±0·09 (30)	15·3±0·09 (34)	13·1±0·12 (32)	−0·6	−1·2
50*	Infiltration	15·7±0·14 (12)	15·8±0·14 (13)	16·6±0·13 (14)	12·9±0·08 (17)	+0·9	−2·9
50	Infiltration	17·2±0·23 (8)	17·1±0·20 (8)	17·0±0·23 (9)	13·5±0·20 (8)	−0·2	−3·6
65	Infiltration	18·5±0·16 (21)	17·6±0·13 (22)	18·7±0·12 (33)	14·1±0·09 (33)	+0·2	−3·5

* Vagi divided on abdominal oesophagus

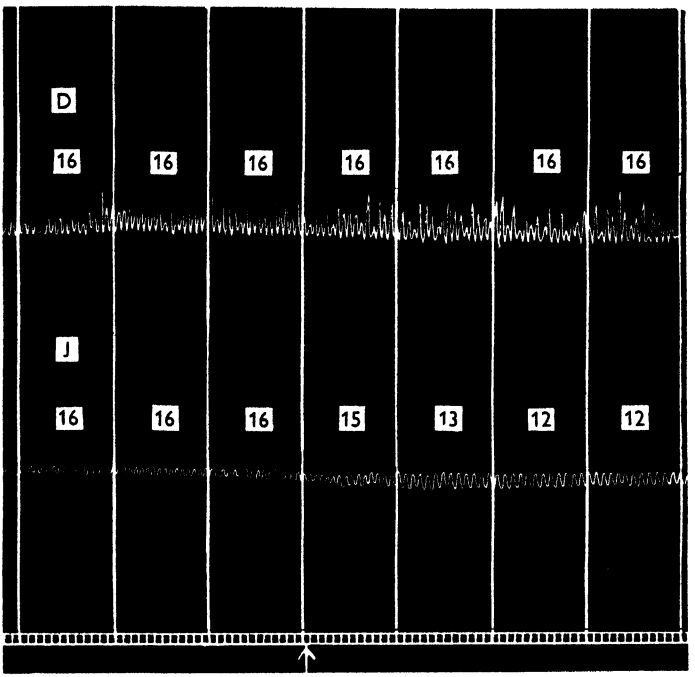

Fig. 6. Contractions of duodenum (D) and jejunum (J) in acute experiment. Time in minutes. At arrow, 10 c.c. of ½% procaine hydrochloride injected round intestinal wall between the balloons. The duodenal rate is unaltered while the jejunal rate falls.

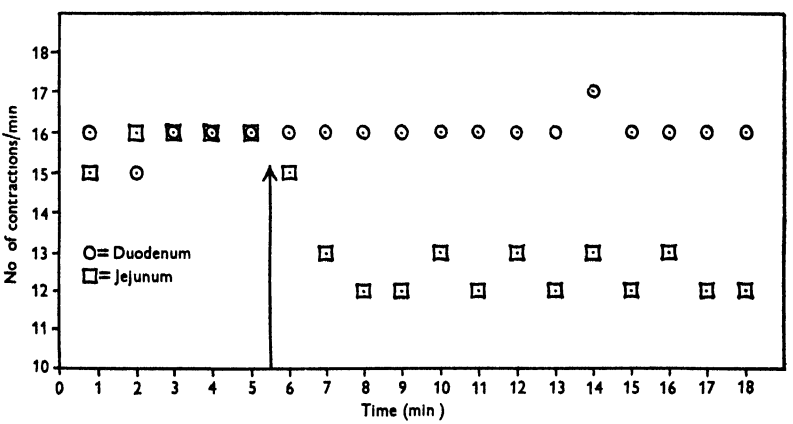

Fig. 7. Rate of contraction of duodenum and jejunum in acute experiment. At arrow, 10 c.c. of ½% procaine hydrochloride injected round intestinal wall between the balloons. The duodenal rate is unchanged while the jejunal rate falls.

Mann & Watkins (1948), in a personal communication, write that they have transplanted loops of jejunum to the ileum in the dog, and found a fall in rate of an order similar to that reported in this paper.

The problem for solution at the outset of this study was why transplantation of a loop of jejunum, with nerve and blood supply intact to lower ileum, should cause a fall in the rate of contraction. The results of both the survival and acute experiments suggest that it is due to division of the intestine proximal to the loop. This step is common to the operations of transplantation and isolation of the segment, and is the only significant operative measure in the operation of proximal section and re-anastomosis. In the acute experiments the injection of the local anaesthetic caused slowing distal to, but never proximal to, the point of infiltration.

The only explanation which appears to fit these facts is that normally the rhythmic activity of the duodenum and jejunum is co-ordinated. When the intestine is transected and re-anastomosed, union takes place by the formation of inexcitable fibrous tissue, and the process of rhythmic excitation cannot then be transmitted to the jejunum. The latter therefore takes up its inherent rhythm in much the same way as do the ventricles when cut off from the atria by division of the bundle of His.

The connexion between atria and ventricles co-ordinates the activity of the heart so that the heart may act as an efficient functional unit. It may be suggested that the rhythmic activity of duodenum and jejunum are similarly co-ordinated so that they may act as a physiological unit as far as motility is concerned. It is of interest that the type of contraction observed in both the duodenum and jejunum consists for the most part of propulsive waves occurring at a rate of 17–18 per min. The contractions of the ileum are different; they occur at the slower rate of 12–14 per min. and commonly have the characteristics of segmentation, that is of alternate contraction and relaxation of adjacent segments without any obvious propulsive properties. These differences in type of contraction are reflected in the different rates of transit of food residues. A barium meal passes more quickly through duodenum and jejunum than through ileum (Shanks, Kerley & Twining, 1938). The slower rate of progress of food residues through the ileum may be related to absorption in the ileum of the chyme which has been thoroughly digested in the more cranial part of the intestine.

It is of interest that Keith (1915) suggested that in the small intestine of the rat there were several rhythmo-genic centres composed of tissue similar to that of the sino-atrial node. He believed that these centres might act as pace-makers for adjacent segments of intestine.

SUMMARY

1. Segments of jejunum, in continuity and with nerve and blood supply intact, were exteriorized and enclosed in bi-pedicled tubes of skin in ten dogs.

2. The rate of rhythmic contraction in a control period of 3–5 weeks was constant in each segment and showed little variation from animal to animal.

3. Transplantation by end-to-end anastomosis of the exteriorized segment to the lower ileum was followed by a fall in rate which persisted for at least a year after operation.

4. A similar fall in rate was seen after isolation of a segment or after transection of the bowel and re-anastomosis proximal to it. A change in rate did not occur after transection of the bowel and re-anastomosis distal to the segment or after hemi-section of the bowel proximal to it.

5. In acute experiments, infiltration of the wall of the intestine with ½% procaine hydrochloride solution was followed by a fall in contraction rate distal to the point of infiltration but by no change proximal to it. Clamping and transection of the bowel had the same effect.

6. These results are taken to suggest that the rate of contraction of the jejunum is influenced by the duodenum.

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VARIABILITY OF THE VITAL CAPACITY OF THE NORMAL HUMAN SUBJECT

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Variations in the vital capacity (v.c.) in different physiological conditions, in health and disease, and between one subject and another, have frequently been recorded. The variations in one subject under supposedly identical conditions seem, however, to have received little attention. A study has therefore been made of control determinations obtained in the course of other investigations.

METHODS AND SUBJECTS

Seventeen healthy male subjects, aged 18–33 years, were used.

Respiratory tracings of v.c. and other determinations were obtained with a Krogh spirometer, and measured to the nearest 10 c.c. All volumes were corrected from spirometer water temperature to 37° C., the assumed temperature in the lungs. To test the supposition that expired air at once assumes the spirometer temperature, the lungs were on several occasions emptied rapidly into the spirometer through a tap, which was immediately closed. Any subsequent cooling of the air would cause a fall in volume, but such shrinkage was never more tham 20 c.c., which is very near the limit of accuracy of the measurements. The conditions in the lungs are much more favourable for thermal equilibration than are those in the spirometer circuit, so it seemed safe to assume that inspired air would promptly reach 37° C. It was also assumed that the air was always saturated with water vapour.

Before measurement of a series of v.c. the subject lay supine on a flat couch for 15 min., and an interval of at least 1½ min. separated successive determinations. This interval should be adequate, as Peabody & Sturgis (1921) carried out measurements at 15 sec. intervals for 10 min., with no progressive change such as might occur with fatigue.

Subjects were given practice at filling and emptying the lungs before any measurements of v.c. were made, and were encouraged to make smooth rather than jerky movements. They were allowed to take their own time, which was usually 15–20 sec. They sometimes supposed that assistance could be obtained by such procedures as bracing the elbows against the couch, or the feet against the lower surface of a table. Such manoeuvres were not usually allowed, but when they were permitted and the effect measured it did not appear that they gave any advantage.

The v.c. was taken as the maximal amount of air which could be expired at the end of a maximal inspiration, or inspired at the end of a maximal expiration

The statistical techniques used are those of Fisher (1941).

RESULTS

Normality of distribution

Despite preliminary practice eight subjects recorded low values for the first two to six determinations of their v.c., later attaining values greater by from 200 to 600 c.c. Such series were disregarded for statistical analysis. Two of these subjects were each re-examined on 2 subsequent days, and now recorded a steady volume for their v.c.

Apart from these series there was always among the measurements made upon one subject on one occasion a scatter which appeared symmetrical, that is, occasional high figures were as frequent as occasional low ones, and the

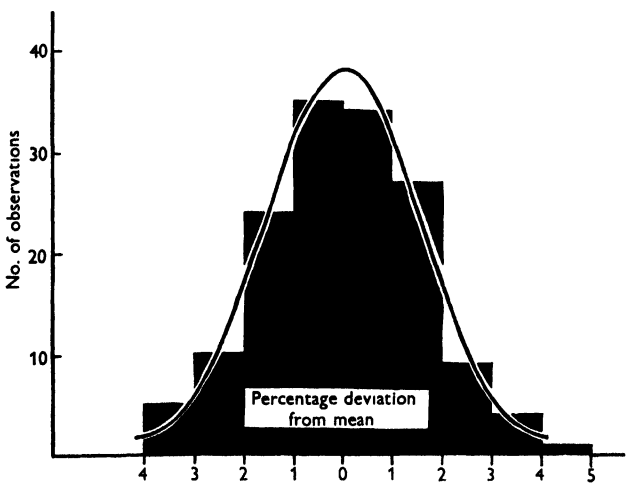

Fig. 1. Distribution of 149 determinations of the vital capacity upon subject P. upon 5 days. Values expressed as a percentage of the mean value for the day in question.

distributions appeared to be approaching normality. A formal test for the normality of distribution of separate determinations on one day has been performed on the subject P., for whom 149 measurements were obtained on five visits. Unfortunately, the means differed significantly from day to day, between 4590 and 5030 c.c., so the values have been calculated as a percentage of the mean for the day in question, and the resulting distribution is shown in Fig. 1. It will be seen that this distribution corresponds closely to the superimposed normal curve, calculated to have the same S.D. and total area, that is, number of observations.

If the distribution were really such that values considerably above the mean were rarer than those correspondingly below the mean, the cubic parameter γ_1 should be negative. The statistic g_1 is, however, $-0{\cdot}042 \pm 0{\cdot}200$, not significantly different from zero, and the distribution is therefore symmetrical.

In fifty-six series of at least ten determinations, upon seventeen subjects,

standard deviations of from 45 to 293 c.c. have been found; and whilst some subjects appear more variable than others, the full range of values has been encounted upon J.N.M. The high values were all in the earlier series, and recent figures have usually been below 100 c.c.

Variability of inspiratory and expiratory depth

To find whether the variations in v.c. were due to varying depth of inspiration or of expiration, the subject expired maximally into the spirometer, connected to a circuit with a total volume of about 8 l., and then inspired maximally; still connected to the spirometer, he breathed quietly for 1¾ min., and then again expired and inspired maximally. In other experiments the sequence was maximal inspiration—maximal expiration—quiet breathing—maximal inspiration—maximal expiration. From each tracing two values for the v.c. were thus obtained, and a series of usually ten such tracings was recorded. The series was discarded unless the initial and final series of v.c. were similar in mean and variance.

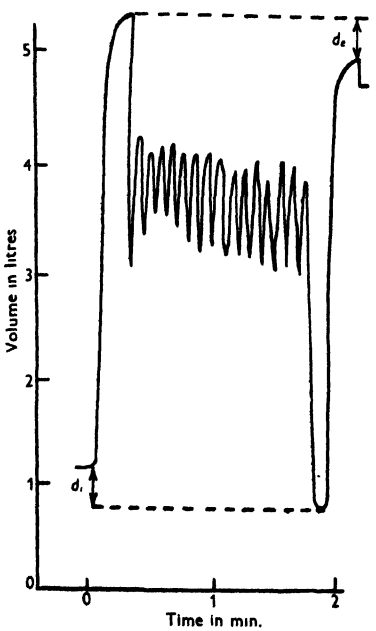

Fig. 2. Spirogram of two successive vital capacity determinations with an interval of 1¾ min., to illustrate procedure and terminology used. d_i, diminution of air volume in spirometer between the two maximal inspirations. d_e, diminution of air volume in spirometer between the two maximal expirations. Expiration upwards.

Fig. 2 shows one of the tracings obtained. Between the two measurements of v.c. some air was lost by respiratory exchange, so the volume recorded for the final maximal expiration or inspiration was less than that for the initial one by an amount designated d_e and d_i respectively. Their means must be equal. If, however, the variation of v.c. is due primarily to varying depth of inspiration or of expiration, the variance of d_i should be considerably greater or less than that of d_e, unless the variation in both is obscured by a very variable air loss through respiratory exchange.

In seven series upon J.N.M. the mean loss of air by respiratory exchange, d_i or d_e, was between 150 and 330 c.c., values which agree well with the respiratory air loss found by rebreathing quietly from the same spirometer circuit for 1¾ min. The S.D. of d_i was 150–320 c.c. and of d_e 24–66 c.c., that is to say, maximal inspiration was much more variable in depth than was maximal expiration. The P value for the ratio of variances in six series was below 0·001 and in one was between 0·01 and 0·001.

Variances similar to those of d_e and d_i respectively were found in series of recordings of two successive maximal expirations or inspirations separated by a few seconds of respiratory relaxation.

Nine other subjects were examined less thoroughly. In two maximal inspiration was significantly more variable than maximal expiration, in one a similar result was doubtfully significant ($P \simeq 0{\cdot}05$), and in six both were equally variable.

Day to day variation

Some series were of the inspiratory v.c., that is, the maximal volume of air that can be inspired after a maximal expiration, and some of the expiratory v.c., and it might be supposed that the inspiratory values would be somewhat greater, since respiratory exchange with a respiratory quotient of less than unity will cause loss of air from the lung-spirometer system during the determination. Upon four occasions with two subjects, inspiratory and expiratory determinations were made alternately, and the mean of the expiratory exceeded the mean of the inspiratory values by 3 ± 29 c.c. (S.E. of difference of means of two series of ten observations each), by 69 ± 71 c.c. (10, 10), by 34 ± 23 c.c. (20, 20) and by -6 ± 32 c.c. (20, 20). It thus appears that any such difference is insignificant.

TABLE 1. Analysis of variance of vital capacity in control determinations, expressed in centilitres

	Degrees of freedom	Sum of squares	Variance	*P**
	Subject J.N.M.			
Between series:				
Due to linear regression on time	1	262,590	—	—
Due to deviations from linear regression on time	32	84,830	2,651	<0·001
Total	33	347,420	10,528	<0·001
Within series	314	85,530	272	—
Total	347	432,950	—	—
	Subject P.			
Between series:				
Due to linear regression on time	1	16,830	—	—
Due to deviations from linear regression on time	3	4,350	1,450	<0·001
Total	4	21,180	5,295	<0·001
Within series	144	8,500	59	—
Total	148	29,680	—	—
	Subjects Fo., Fr., L., Pa., R. and T.			
Between series upon the same subject	8	4,221	528	<0·001
Within series	206	26,365	128	—

* Probability that variance is really identical with that within series.

When two or more series of determinations were made on the same subject a few days or weeks apart, their means often differed significantly, and tended to increase. Analyses of variance, shown in Table 1, were therefore carried out on the two principal subjects (J.N.M., 348 observations in thirty-four series;

P., 149 observations in five series), including regression analysis on time in days. A similar analysis of variance of the pooled data on six other subjects (215 observations in fourteen series) is also included in the table. It will be seen that the differences between series are highly significant compared with the differences between observations within series. The differences between series consist of two components, a linear regression on time and deviations from linear regression, both highly significant. This means that there was a significant tendency to steady increase of v.c. with time (accounting for about three-quarters of the variation between series) and also a significant and as yet unexplained day-to-day variation in v.c. which is independent of time. With both subjects there was a small but significant negative correlation between v.c. and room temperature, but the fitting of partial regression functions on time and temperature did not greatly reduce the residual variance from regression.

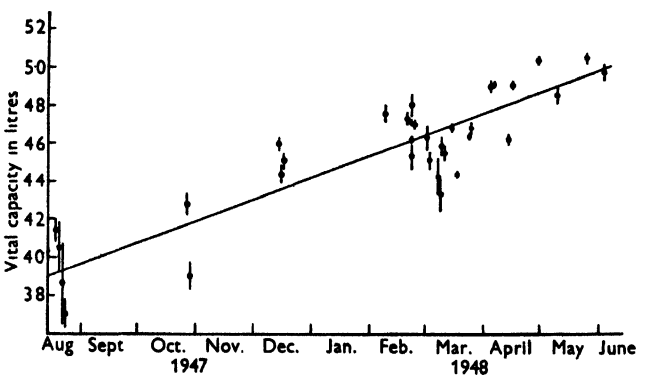

Fig. 3. Subject J.N.M. Mean vital capacity on different dates, with E.S.D. of each mean indicated by vertical strokes, and linear regression of vital capacity upon date inserted.

The steady rise of v.c. in J.N.M., about 1 l. in 9½ months, is shown in Fig. 3. P.'s v.c. increased by about 450 c.c. over an 18-day period.

DISCUSSION

Very few figures have been found which show the normal variation in v.c. determinations on one subject. Christie (1932) states that even under carefully controlled conditions duplicate determinations may differ by several hundred c.c. Sjöstrand (1941) has published a series of fifty-five triplicate determinations upon two subjects under a wide variety of conditions, and the S.D. calculated from his pooled results is 56 c.c., but the temperature and pressure at which his volumes are expressed is not stated. Gilson & Hugh-Jones (1949) have found a S.D. of between 85 and 140 c.c. in five normal and seven abnormal subjects. These figures are very similar to those here reported, and the narrower range may be due to the smaller number of series of observations.

The determinations upon one subject on one occasion are normally distributed, as was also found by Gilson & Hugh-Jones, and there is thus no indication that the larger values approach any maximal value which could be described as the 'true' v.c. The lungs are apparently stretched sometimes more, sometimes less, by more or less effective muscular efforts. There is thus no rational justification for publishing the result of two or more determinations as the maximal instead of the mean value, as West (1920), for example, has done, unless a progressive increase in volume suggests improvement with practice.

With J.N.M. the random variation is much greater for maximal inspiratory than for maximal expiratory efforts, and there is some indication that the same is true with some other subjects. Several authors (Christie, 1932; Hurtado & Boller, 1933; Gilson & Hugh-Jones, 1949) have commented upon a different variability in the reserve or complemental air, but unless the tidal air remained constant these observations do not necessarily represent variations in the depth of maximal expiratory or inspiratory efforts. The objective evidence is at complete variance with the subjective impression, that when expiring maximally it is usually possible to expel a little more air by an even more powerful effort, but that when inspiring maximally one reaches a fixed point beyond which no more can be inspired.

A progressive increase of v.c. with time, here observed in two subjects, was also noted by Gilson & Hugh-Jones, and suggests a slow improvement with repeated practice, even over a considerable period of time. The quite large residual variance from regression might be due to variations in pulmonary congestion, through variations in vasomotor tone in the systemic circuit. Varying degrees of distension of the abdominal viscera are not likely to be responsible, as most series of determinations were performed at about the same time after a similar breakfast, and Mills (1949) has shown that considerable abdominal distension has little effect upon the v.c. The occurrence of such day to day variation stresses the need for adequate control of all observations upon alterations in v.c.

SUMMARY

1. Sixty-two series of control determinations of the vital capacity have been performed upon seventeen subjects, comprising 839 separate determinations. Upon any one subject on any one occasion, the values are normally distributed, with unusually high values as frequent as unusually low ones. The S.D. usually lies between 50 and 200 c.c.

2. The variation in one series is due to more or less successful muscular effort, and the maximal inspiratory effort is often more variable than the maximal expiratory effort.

3. Variance analysis shows a considerable day to day variation in the v.c. outside the variation to be expected on one occasion. In the two subjects

repeatedly examined, there has been a continuous increase, extending in one subject over 9½ months and amounting to about 1 l.; variance from regression on time is, however, highly significant.

My thanks are due to those who have acted as subjects for these experiments, and to Mr S. Langford for technical assistance.

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THE INFLUENCE OF ABDOMINAL DISTENSION UPON THE VITAL CAPACITY

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The reduction of vital capacity (v.c.) on lying down has in the past been ascribed to the pressure exerted on the diaphragm by the abdominal contents. This explanation is not now generally accepted; but beyond the single observation by Asmussen, Christensen & Sjöstrand (1939) that introduction of 500 c.c. of water into the stomach does not alter the v.c., no mention has been found of the effect upon v.c. of varying the contents of the abdomen. Since there is a considerable day to day variation in the v.c. (Mills, 1949), and since the contents of the abdomen will vary from time to time, the possible connexion between these two variables has been investigated.

METHODS AND SUBJECTS

The author, aged 34, and seven other healthy males, aged 18–20, were used as subjects.

Tracings of the v.c., taken as the maximal amount of air which can be expired after a maximal inspiration, were made with a Krogh spirometer and measured to the nearest 10 c.c. All volumes are expressed at 37° C. saturated.

The subject lay supine upon a flat couch throughout each experiment, except when he rolled on to his side to void urine. Ten control records of v.c. were made on each occasion, 2½ hr. or more after a light or no breakfast and after a rest of 15 min. on the couch, during which new subjects were given practice at emptying and filling the lungs. Then another ten records were made, either after the introduction into the stomach, sometimes by drinking, sometimes through a Ryle's tube inserted via the nose, of 1 or 2 l. water at about 37° C., or else after eating 1 kg. of equal parts of apples and sandwiches. Sometimes a second litre of water was introduced, or fluid was aspirated from the stomach, or urine was voided by the subject without rising. Any further change in v.c. was measured from ten more records.

RESULTS

The mean control v.c. for different subjects lay between 3940 and 5560 c.c., and the S.D. between 35 and 156 c.c. The changes produced are shown in Table 1, where it will be seen that they were inconstant and usually small. One litre of water was often, and 2 l. of water or 1 kg. of food was always, enough to leave the abdominal wall distinctly uncomfortable and sore. It is thus possible that some of the reductions of v.c. were due not so much to inability as to unwillingness to make maximal respiratory movements.

TABLE 1. Change in vital capacity in c.c. when water at 37° C. or food enters or leaves the stomach. All changes are differences between means of ten control and ten test determinations except where indicated in parenthesis. S.E. of each difference of means is given.

Subject	Change when 1 l. water enters stomach	Futher change when second litre water enters stomach	Volume of liquid leaving stomach and bladder* (c.c.)	Resultant change
J.N.M.†	−29±42	—	—	—
J.N.M.†‡	−144±50	+49±54	—	—
J.N.M.‡	−108±34	−140±28	U 865 G 325	+67±40
J.N.M.‡	−98±42	−43±44	G 810	+53±25
F.	+7±36	−201±39	—	—
D.	+87±41	−155±46	—	—
D.	+11±42	−82±54	—	—
G.	−17±24	+12±34	—	—
Mo.	−49±98	—	—	—
Ma.	+306±34	−72±24	—	—
K.	−24±49	−166±50 (10, 7)	—	—
W.	+5±28	−218±37	—	—
	Change when 2 l. water enters stomach			
J.N.M.†	−338±70	—	U 725	+1±52
J.N.M.‡	−177±27	—	G 930	+66±26
J.N.M.‡	−183±46 (10, 9)	—	G 1100	+180±31 (9, 10)
	Change when 1 kg. food is eaten			
J.N.M.	+189±66	—	—	—
J.N.M.	+14±56	—	—	—

* U indicates urine. G indicates liquid aspirated from stomach.
† Water at room temperature entered stomach.
‡ Water admitted through Ryle's tube; in other experiments water was drunk.

Some water of course left the stomach rapidly. After the introduction of 2 l. of water into J.N.M.'s stomach, volumes of 1100, 930 and 810 c.c. were recovered through the Ryle's tube after 40, 41 and 53 min., but some of the residue may still have been in the intestinal lumen. The experiments were therefore performed as speedily as possible. Subjects other than J.N.M. commonly drank even their second litre in less than 2 min., and the complete experiment, from starting to drink the first litre to the end of ten v.c. recordings after the second litre, occupied 20–25 min.

The experiments with solid food were intended to produce a more lasting gastric distension and the gastric contents were presumably further increased by secretion of juice. The eating occupied 33 and 35 min. and the v.c. recordings were completed in about 7 min.

In a few further experiments upon J.N.M., rapid filling and emptying of the stomach was achieved by passing a tube with internal diameter 5 mm., which allowed 1 l. of water to enter the stomach in ½ min. In twenty-eight such experiments a mean of 834 c.c. (range 750–950 c.c.) was recovered through the

tube 5 min. or so later. This wide gastric tube left the mouth through a side opening in the respiratory tubing, so that tracings could continue while water was run into or out of the stomach. Pairs of v.c. were thus recorded, with an interval of 1¾ min. rebreathing from the spirometer during which either 1 l. of water was run into the stomach, or the stomach was drained, or nothing was done (controls). The v.c. was not significantly altered by any of these procedures (ten performances of each). The diminution of spirometer vol. from first to second maximal inspiration or expiration was the same ($P > 0{\cdot}3$) in all three sets of experiments, so it is clear that 1 l. of extra fluid in the stomach neither impeded inspiration nor assisted expiration, and the loss of air was presumably due to respiratory exchange.

DISCUSSION

Even if some administered fluid was absorbed before v.c. determinations were finished, it is clear from these experiments that large changes in abdominal contents produce small and inconstant changes in v.c. J.N.M.'s v.c. fell little if at all when 1 l. water entered the stomach, that of other subjects never fell significantly and occasionally rose. Two litres of water usually reduced the v.c., but not greatly, although the abdomen felt uncomfortably full.

TABLE 2. Analysis of variance of vital capacity of J.N.M., expressed in centilitres

	Degrees of freedom	Sum of squares	Variance	P*
Between control and test series on one day	17	15,283	899	<0·001
Between days, controls only	6	5,950	992	<0·001
Within series	215	20,054	93	—

* Probability that variance is really equal to that within series.

The variations produced have, for J.N.M., been compared with the random day-to-day variation of controls by the variance analysis shown in Table 2. Recordings taken on each of 7 days provide a control series made with the stomach empty, and two or three series made either immediately after the addition or removal from the stomach of large volumes of liquid or at 20 min. interval after the addition, by which time liquid had presumably left the stomach and had entered the bladder in considerable amount. It is shown that the variation induced in the v.c. by running 1 or 2 l. of water into or out of the stomach is comparable with, and slightly less than, the random variation between control determinations on different days, and it is therefore unlikely that the much smaller variations in abdominal contents which may occur in controlled conditions contribute appreciably to the day-to-day variation found in the v.c. by Mills (1949).

Sufficient abdominal distension might be expected to limit maximal inspiration and possibly to assist maximal expiration. It appears, however, that 1 l.

of fluid added to the stomach has neither effect, and hence that maximal inspiration is not normally limited by resistance to the downward movement of the diaphragm.

SUMMARY

1. The vital capacity shows small and inconstant changes on addition of 1 l. water or 1 kg. food to the empty stomach, but usually falls slightly on addition of a second litre, and rises slightly when the stomach is drained.

2. The variation induced in the vital capacity by running 1 or 2 l. of water into or out of the stomach is comparable with the day-to-day variation between controls on an empty stomach.

3. One litre of extra fluid in the stomach neither restricts inspiration nor assists expiration.

My thanks are due to those who have acted as subjects, and to Mr S. Langford for technical assistance.

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ACTION OF POTASSIUM IONS ON BRAIN METABOLISM

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Potassium ions cause pronounced effects on the metabolism of glucose by slices of cerebral cortex, increasing aerobic glycolysis and respiration and inhibiting anaerobic glycolysis (Ashford & Dixon, 1935; Dickens & Greville, 1935). Gerard (1938) suggested that potassium may leak out of neurones during asphyxia and stated that he had some evidence in support of this contention (see also Cowan, 1934). Gerard further supposed that potassium released in asphyxia might then cause increase in respiration and stimulation of nervous activity. Dixon (1940) considered that liberation of potassium ions with attendant inhibition of anaerobic glycolysis might be responsible for the final paralytic symptoms which follow cerebral ischaemia. Recently it has been found (Dixon, 1949) that K leaks out of brain when it is deprived of both glucose and oxygen. Leakage of K from brain also occurs during convulsions (Colfer & Essex, 1947). Liberation of K from brain cells may thus take place in various forms of cerebral disease and it seems possible that such liberated K ions might then produce secondary toxic effects on brain metabolism. It is therefore of interest to know more precisely the conditions necessary to produce these metabolic effects and the rapidity with which they are exerted.

In the experiments of Ashford & Dixon with slices of rabbit cerebral cortex potassium chloride was added to the Ringer before the start of the experiments. Dickens & Greville, however, in some cases added solid KCl during the course of their experiments to the Ringer solution in which slices of rat cerebral cortex were suspended. Respiration (in phosphate-Ringer) was increased after 10 min., and anaerobic glycolysis (in bicarbonate-Ringer) was diminished during a 30 min. period following the addition. Owing to pressure changes produced by the addition of solid KCl a lapse of 10 min. occurred before metabolic observations could commence. To eliminate this difficulty the rapidity of onset of the K effect has now been studied by adding KCl already in solution. The present communication records continuous manometric observations on the glycolysis of slices of rabbit cerebral cortex before and after the addition of small volumes of KCl in aqueous solution to the bicarbonate saline in which the slices were

immersed. In the experiments of Ashford & Dixon (1935) and Dixon (1940) effects were observed with concentrations of added K as low as 0·02 M, but 0·1 M was the usual concentration employed. An attempt was therefore made to define more precisely the concentrations of added potassium necessary to produce the main alterations in metabolism. Some experiments have also been made to decide whether the K effect can occur under isotonic conditions when the saline solution used is diluted by an amount equivalent to the K added. Substantially the same results were obtained. It is thus apparent that the effects of K on metabolism may well take place under conditions realizable *in vivo* in living tissues.

METHODS

Slices of rabbit cerebral cortex were used throughout. The glycolysis was measured with the tissue immersed in glucose-bicarbonate-Ringer of the same composition as that described by Dickens & Greville (1935). This solution contains in g./100 ml. NaCl 0·7, KCl 0·018, $CaCl_2$ 0·019, $MgCl_2$ 0·0076, $NaHCO_3$ 0·21, and glucose 0·2; it was made up daily from stock solutions of the following composition in g./100 ml.:

Stock solution *A*	Stock solution *B*	Stock solution *C*
NaCl 7	$NaHCO_3$ 2·1 saturated with CO_2	Glucose 2 made up daily
KCl 0·18		
$CaCl_2$ 0·19		
$MgCl_2$ 0·076		

2 ml. of *A*, 2 ml. of *B* and 2 ml. of *C* were added to a graduated glass-stoppered measuring cylinder and diluted to 18 ml. The mixture was then equilibrated with N_2 or O_2 containing 5% CO_2. Of this solution 1·8 ml. was placed in the main cup of each Warburg manometer and 0·2 ml. of water, KCl solution or NaCl solution was placed in the side bulb. The slices of rabbit brain were then immersed in the fluid in the main cups and each manometer was filled with N_2 or O_2 containing 5% CO_2. The manometers were then shaken in a bath at 38° and readings were commenced after 10 or 15 min. Thermobarometers containing the same fluid and gas as used in the cups containing the brain slices showed that equilibration had been reached by the time readings were commenced. No significant pressure changes followed the addition from the side bulbs of 0·2 ml. of water, KCl solution, or NaCl solution, to the 1·8 ml. of fluid in the main cups of the thermobarometers. When constant readings had been recorded over a few 10 or 15 min. intervals, the fluid contents of the side bulbs were tipped into the main cups. After the dilution of the 1·8 ml. of fluid in the main cups by the 0·2 ml. of water from the side bulbs the concentrations of salts and glucose in the final mixture were the same as described above in the glucose-bicarbonate-Ringer of Dickens & Greville. Before this addition the solutions were slightly stronger. No metabolic change was however detected following this dilution by 10% extra water, the glycolytic rates before and after being identical. In the parallel experiments in which KCl and NaCl solutions were added, these salts were dissolved in the 0·2 ml. fluid in the side bulbs in such amount as to give the varying required final concentrations of these added ions after dilution to 2 ml. by the 1·8 ml. of fluid in the main cup. This technique obviated the difficulty of adding solid KCl or NaCl.

The slices of rabbit cerebral cortex were washed with Ringer's solution before being placed in the cups. In the first experiments the washing fluid was made by diluting stock solution *A* 1:10 with water. This solution contained no bicarbonate and was thus slightly hypotonic. In later experiments either bicarbonate Ringer or the full glucose bicarbonate Ringer was used for washing the slices. Results were unaffected by using these salines of slightly different composition for the preliminary washing.

Anaerobic glycolysis was evaluated by manometric measurement of the CO_2 evolved. Aerobic glycolysis was estimated on the assumption that the fall in pressure due to respiratory absorption of oxygen cancels the pressure change due to the formation of respiratory CO_2. This is only approximately true with small volumes of fluid (2 ml. was used), and it is realized that the figures for aerobic glycolysis are probably on the low side. The comparative value of the figures for aerobic glycolysis is probably little diminished by this approximation. Furthermore, results obtained by this method were nearly the same as those obtained simultaneously with Warburg's two-vessel method (see Table 4).

RESULTS

Many of the results are expressed graphically (Figs. 1–6), but in some cases numerical values of anaerobic glycolysis ($Q_L^{N_2}$) and aerobic glycolysis ($Q_L^{O_2}$) are given (Tables 1–5).

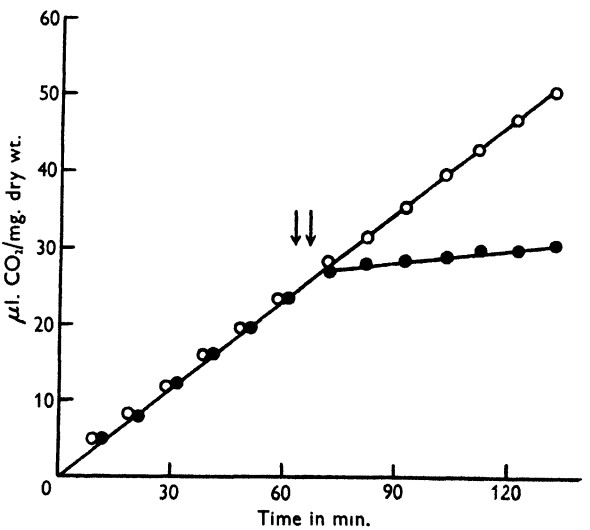

Fig. 1. Effect of addition of K on rate of anaerobic glycolysis. Both series of points record normal anaerobic glycolysis in two parallel experiments up to the period between the arrows. Here 0·2 ml. of water was added in curve ○—○—○, and the same volume of KCl solution of such strength as to give a final concentration of 50 m.equiv./l. of added K in the curve ●—●—●.

Fig. 1 and Table 1 show the effect of the addition of KCl on the anaerobic glycolysis of rabbit cerebral cortex. Using samples from the same brain, in seven parallel experiments, normal anaerobic glycolysis was observed during a preliminary control period. Then various amounts of KCl, dissolved in 0·2 ml. of water, were added to the 1·8 ml. of glucose-bicarbonate-Ringer containing each sample of the brain slices. In Fig. 1, for the sake of clarity, only the effects of adding 0·2 ml. of pure water and 0·2 ml. 0·5 N-KCl are recorded. In the former case the K concentration was not increased, and in the latter case the K concentration was increased by 50 m.equiv./l. The rate of anaerobic glycolysis from the start of the experiments up to the time of the addition was constant and was not affected to any detectable degree by the

addition of 0·2 ml. of water. Normal anaerobic glycolysis is in fact, under the conditions studied, a remarkably constant process during the whole period of incubation. The addition of 50 m.equiv. K/l. caused rapid inhibition of anaerobic glycolysis, which was well established (Fig. 1) within the first 10 min. period following the addition. Table 1 shows the changes produced in the quotients of anaerobic glycolysis by the varying amounts of KCl solution in this series of experiments.

TABLE 1. Anaerobic glycolysis before and after addition of varying amounts of KCl. in aqueous solution (seven parallel experiments with slices from same rabbit brain)

$Q_L^{N_2}$ before addition of KCl solution		$Q_L^{N_2}$ after addition of KCl solution		Final concentration of added KCl m.equiv./l.
1st ½ hr.	2nd ½ hr.	3rd ½ hr.	4th ½ hr.	
24	23	22	22	0
23	23	24	23	10
23	24	16	13	25
30	28	9	7	30
26	24	6	5	40
25	23	3	4	50
25	24	1	3	100

First ½ hr. period commenced 20 min. from start of incubation. KCl solutions (water in control) were added to each cup from side bulb between the end of the 2nd and beginning of 3rd ½ hr. period over a period of 4 min. (63–67 min. from the start of 1st ½ hr. period). The 3rd ½ hr. period began 5 min. later.

The symbol $Q_L^{N_2}$ represents anaerobic glycolysis measured in μl. CO_2/mg. dry wt. of tissue/hr. in N_2. The lactic acid formed by glycolysis liberates an equivalent amount of CO_2 from the bicarbonate-Ringer.

Fig. 2 shows graphically (in another series of parallel experiments) the effects of adding various amounts of KCl in solution so as to raise the final concentrations of K by 10–100 m.equiv./l. The level for a marked effect lies between 20 and 30 m.equiv./l. (see below). The inhibition was again rapid even at the lower effective concentrations, and could be measured during the first 10 min. interval after that in which K was added. More rapid inhibition could scarcely be detected by the manometric technique.

Fig. 3 and Table 2 refer to a similar series of parallel experiments in which isoequivalent quantities of NaCl were added in the control experiments instead of pure water. In each case 40 m.equiv./l. of total alkali metal ions were added. Na alone produced no effect, but profound and rapid inhibition was observed after the addition of 30 m.equiv. of K/l. With 20 m.equiv./l. of added K some inhibition was seen in this case. In a more recent series, after additions of 50 m.equiv./l. of Na, the glycolysis remained at 94% of the original in each of two parallel experiments. However, after the addition of 50 m.equiv./l. of K only 12 and 14% of the initial glycolysis remained in a pair of simultaneous duplicate experiments with samples of this same tissue. Here NaCl produced

no effect, but KCl rapidly inhibited glycolysis within 6–9 min. In this series other additions were not made.

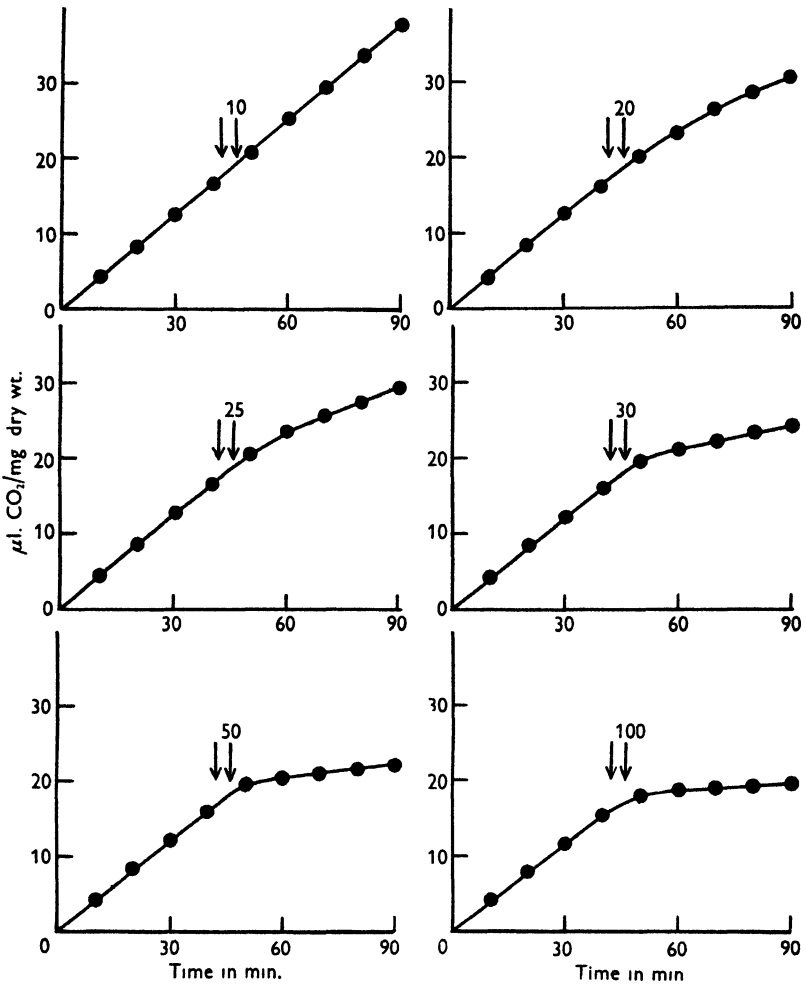

Fig. 2. Effects of varying additions of K on rate of anaerobic glycolysis. Each curve shows anaerobic glycolysis in parallel experiments up to period between arrows. At this point KCl solutions were added to give final concentrations of added K in m.equiv./l. indicated by number over right-hand arrow.

Fig. 4 summarizes the results of the above and other experiments. It shows anaerobic glycolysis after the addition of KCl expressed as percentage of the initial anaerobic glycolysis before addition, plotted against the concentration of added K in m.equiv./l. The results were obtained from six series of parallel experiments using in each series the brain of a different rabbit. In three cases

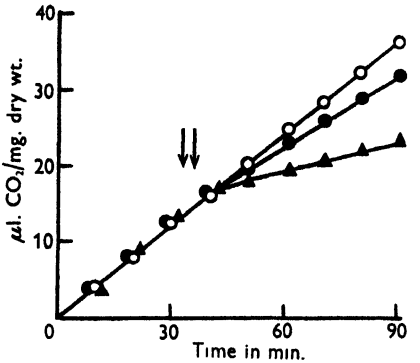

Fig. 3. Effect of addition of Na and K on rate of anaerobic glycolysis. Each series of points records normal anaerobic glycolysis up to period between arrows. Here NaCl and KCl were added in solution in such concentration as to give the following final strengths of each salt in m.equiv./l. ○—○—○, NaCl 40, KCl 0; ●—●—●, NaCl 20, KCl 20; ▲—▲—▲, NaCl 10, KCl 30.

TABLE 2. Anaerobic glycolysis before and after addition of varying amounts of KCl and NaCl in aqueous solution (five parallel experiments with slices from the same rabbit brain)

$Q_L^{N_2}$ before addition of salts in solution 1st ½ hr.	$Q_L^{N_2}$ after addition of salts in solution: 2nd ½ hr.	3rd period (20 min.)	Final concentration of added salts m.equiv./l.: KCl	NaCl
25	25	24	0	40
27	29	24	10	30
25	19	18	20	20
26	8	7	30	10
29	8	4	40	0

First ½ hr. period commenced 15 min. from start of incubation. Salt solutions were added to each cup from side bulb between end of 1st and beginning of 2nd ½ hr. period over a period of 3 min. (33–36 min. from the start of 1st ½ hr. period). The 2nd ½ hr. period started 4½ min. later.

NaCl was added simultaneously with the KCl so as to produce an equimolar rise in total salt concentration in parallel experiments. In the other cases KCl only was added. The glycolysis was measured over a 20 or 30 min. period before the addition of KCl, and again over a 30 min. period after the addition neglecting the glycolysis during the 10 min. period in which the addition was made. The main inhibition of glycolysis was established between 20 and 40 m.equiv./l. of added K. Further increases produced little additional effect.

The rapidity of the KCl effect on aerobic glycolysis has also been studied. Here the aerobic glycolysis has been evaluated approximately (see above) from the pressure changes produced in manometers filled with O_2 95%, CO_2 5% and containing 2 ml. of fluid. Fig. 5 records some of the results from a series

of five parallel experiments in which Na and K salts in solution were added in such amounts as to produce in each case an increase by 40 m.equiv./l. in the concentration of total alkali metal ions. No effect was observed with NaCl alone nor with 10 nor 20 m.equiv./l. of K. With the addition of 30 m.equiv./l. of K, a definite increase in aerobic glycolysis was produced, and with 40 m.equiv./l. of K intense aerobic glycolysis developed with such rapidity that the full rate was reached within 10 min. of adding the KCl solution. For the sake of clarity only three of these parallel experiments are recorded in Fig. 5. Other similar series of experiments have given the same results.

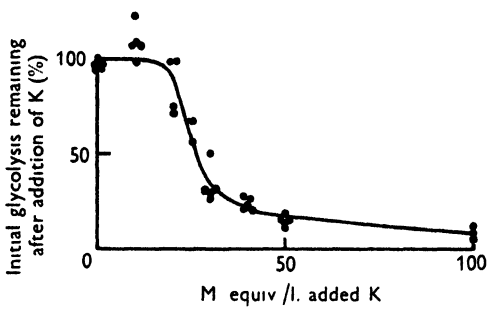

Fig. 4. Relation of inhibition of anaerobic glycolysis to concentration of added K.

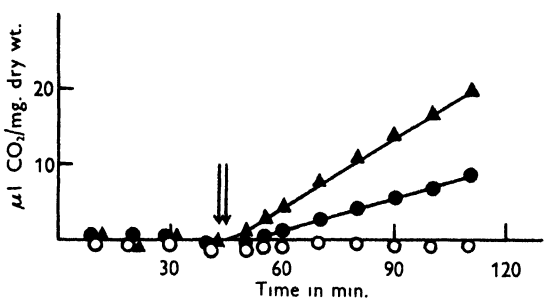

Fig. 5. Effect of addition of Na and K on the rate of aerobic glycolysis. Each series of points records normal aerobic glycolysis up to the period between the arrows. At this point NaCl and KCl were added in solution in such concentration as to give the following final strengths of each salt in m.equiv./l.: ○—○—○, NaCl 40, KCl 0; ●—●—●, NaCl 10, KCl 30; ▲—▲—▲, NaCl 0, KCl 40.

Fig. 6 and Table 3 show the effect of adding varying amounts of KCl solution alone on the aerobic glycolysis in another series of five parallel experiments. A definite increase in aerobic glycolysis first became evident with 30 m.equiv./l. of added K. With 50 m.equiv./l. of added K the aerobic glycolysis had reached the normal high anaerobic rate (Table 3). The full rate of enhanced glycolysis was attained in each case within 6–8 min. of the addition of KCl. Owing to the method used, however, small increases of aerobic glycolysis at lower concentrations may have escaped detection.

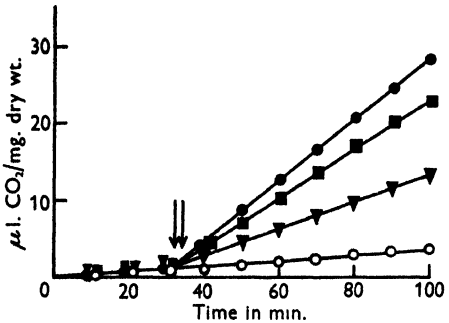

Fig. 6. Effect of addition of K on rate of aerobic glycolysis. Each series of points records normal aerobic glycolysis up to the period between the arrows. At this point KCl was added in solution in such concentration as to give the following final strengths of K in m.equiv./l. ○—○—○, KCl 20; ▼—▼—▼, KCl 30; ■—■—■, KCl 40; ●—●—●, KCl 50.

TABLE 3. Approximate values of aerobic glycolysis before and after the addition of varying amounts of KCl in aqueous solution (five parallel experiments with slices from the same rabbit brain)

$Q_L^{O_2}$ before addition of KCl solution 1st ½ hr.	$Q_L^{O_2}$ after addition of KCl solution 2nd ½ hr.	3rd ½ hr.	Final concentration of added KCl m.equiv./l.
2	3	3	20
3	10	11	30
3	19	19	40
3	25	24	50
3	21	18	100

First ½ hr. commenced 12 min. from start of incubation. KCl solutions were added to each cup from side bulb between the end of the 1st and beginning of the 2nd ½ hr. period over a period of 2 min. (32–34 min. from the start of the 1st ½ hr. period). The 2nd ½ hr. period started 6 min. later. The symbol $Q_L^{O_2}$ represents aerobic glycolysis measured in μl. CO_2/mg. dry wt. of tissue/hr. in O_2. The lactic acid formed by glycolysis liberates an equivalent amount of CO_2 from the bicarbonate-Ringer. It is assumed that the pressure change due to the formation of CO_2 in respiration is cancelled by the respiratory absorption of O_2. This approximation gives useful comparative values which appear to be only slightly smaller than those obtained by Warburg's two vessel method (see Table 4).

In the experiments so far described KCl was added in addition to the normal constituents of the Ringer solution. It might be argued that these ionic effects on metabolism thus depend on the final solution being hypertonic. This point was raised by Dickens & Greville (1935) who found that with rat brain the effect of addition of 0·1 M-KCl was largely eliminated when an equivalent amount of NaCl was simultaneously subtracted from the Ringer solution. To test this question further some experiments have now been made in which the Ringer solution was diluted by an amount equivalent to the added K. Since an addition of 0·04 N-KCl produces practically the full effect it was decided to add this amount of KCl with simultaneous dilution of the other metallic

chlorides of the Ringer. This would not cause such a profound modification of Na content as the addition of as much as 0·1 N-KCl with corresponding subtraction of other salts. The Ringer solution normally used (see Dickens & Greville, 1935) is 0·1275 N with respect to metallic chlorides. In these experiments 69 % of the usual amount of stock concentrated Ringer solution was employed. Thus the normality of the final solution would be diminished by 0·04 N. To offset this dilution one-tenth of the final volume of 0·4 N-KCl was added, thereby raising the final concentration of KCl by 0·04 N (actually 0·039 N when correction is made for initial dilution of KCl originally present in the stock concentrated Ringer solution). At the same time the total salt concentration of the final solution is maintained at isotonic level. The ratio of bivalent to univalent ions in these solutions is so small that their osmotic pressure may be taken as proportional to their normality (within 1 %). The dilution of Ca and Mg involved in this change was not responsible for metabolic effects, since controls in which 0·04 N-NaCl instead of 0·04 N-KCl was added gave substantially normal glycolytic quotients. The results obtained are recorded in Table 4.

TABLE 4. Anaerobic and aerobic glycolysis in the presence of added NaCl and KCl under isotonic conditions (glucose content 0·2 % and bicarbonate 0·025 M as previously)

Brain cortex slices from rabbit no.	Normal Ringer solution		Diluted Ringer solution + NaCl		Diluted Ringer solution + KCl			
	$Q_L^{N_2}$	$Q_L^{O_2}$	$Q_L^{N_2}$	$Q_L^{O_2}$	$Q_L^{N_2}$	$Q_L^{O_2}$	$Q_L^{O_2}W$	Q_{O_2}
1	23	2	—	—	5	22	—	—
2	26	1	—	—	—	26	29	−40
3	—	—	26	2	4	22	23	−21
4	—	—	28	3	4	24	26	−29

Normal Ringer: stock solution *A* (conc. Ringer solution) 2 ml.; 0·25 M-$NaHCO_3$, 2 ml.; 2 % glucose, 2 ml.; diluted to 20 ml. *Diluted Ringer* + NaCl· (The use of this control was kindly suggested to me by Dr G. D. Greville): stock solution *A*, 1·37 ml.; 0·25 M-$NaHCO_3$, 2 ml.; 0·4 M-NaCl, 2 ml.; 2 % glucose, 2 ml.; diluted to 20 ml. *Diluted Ringer* + KCl: stock solution *A*, 1·37 ml.; 0·25 M-$NaHCO_3$, 2 ml.; 0·4 M-KCl, 2 ml.; 2 % glucose, 2 ml.; diluted to 20 ml.

The substitution of 1·37 ml. instead of the normal 2 ml. of stock solution *A* entails a dilution of the final solution by 0·04 equiv. of total salts per l. This subtraction is equivalent to the added Na or K.

The quotients $Q_L^{N_2}$ and $Q_L^{O_2}$ are used as defined in footnotes to Tables 1 and 3. Q_{O_2} and $Q_L^{O_2}W$ indicate respectively the quotients for oxygen uptake and lactic acid production observed in experiments using the two-vessel method of Warburg. The simplified method of calculating lactic acid production indicated by $Q_L^{O_2}$ (using one vessel) gives very similar values to $Q_L^{O_2}W$ found by the two-vessel method.

From Table 4 we see that the addition of 40 m.equiv./l. of KCl produced its full effect in inhibiting anaerobic glycolysis and stimulating aerobic glycolysis even when an equivalent subtraction was made from the other salts. Similar dilution followed by addition of 40 m.equiv./l. of NaCl produced no such effect. This addition of KCl in isotonic solution also gave very high values for

respiration. It is thus apparent that the effect of K ions on metabolism do not depend on the presence of a hypertonic saline environment and may thus take place under conditions likely to be realized *in vivo*.

Irritation followed by paralysis is a common sequence of events in cerebral disease. Stone (1938) suggested that convulsions (including those of cyanide poisoning) and increased activity might result in increased lactic acid production, whereas narcosis and diminished activity might inhibit glycolysis. Dixon (1939, 1940) supposed that increase in glycolysis might generate the convulsions and irritative phenomena which occur initially in cerebral anoxia, and that subsequent inhibition of glycolysis would account for the later paralytic symptoms which are the final outcome of interruption of blood supply. This final inhibition of glycolysis would result directly from defective supply of glucose to the ischaemic area, and also possibly indirectly from the liberation of K which takes place when brain is deprived of both glucose and oxygen. Such liberated K could cause rapid and profound inhibition of glycolysis in anaerobic regions still supplied with glucose. In regions with full aeration, on the other hand, a rapid increase in glycolysis above normal would be anticipated, which might lead to excessive activity at the periphery of the damaged area (see Gerard, 1938).

TABLE 5. Irreversibility of effect of K on anaerobic glycolysis

Concentration of added KCl (m.equiv./l.)	0	10	20	30	50
$Q_L^{N_2}$ in Ringer solution containing this added KCl	25	34	11	7	5
$Q_L^{N_2}$ of same slices of rabbit brain after transference to normal Ringer solution:					
1st 30 min.	21	21	11	10	—
2nd 30 min.	20	20	10	8	7

The anaerobic inhibition of glycolysis by K is probably irreversible. Ashford & Dixon (1935) showed that the effect of 0·1 M-KCl on anaerobic glycolysis was not removed by subsequent immersion of the brain slices in normal Ringer. Table 5 shows this irreversible effect of added KCl with concentrations down to 0·02 M. It is thus possible that even a transitory increase in K concentration under anaerobic conditions may be followed by lasting interference with the cellular glycolytic system and concomitant irreversible damage to the neurones.

SUMMARY

1. The rapidity of onset and the effective concentration limits of the action of potassium ions on brain glycolysis were studied.

2. Inhibition of anaerobic glycolysis became evident at about the level of 20 m.equiv./l. of added potassium. With 40 m.equiv./l. of potassium 75% of the anaerobic glycolysis was suppressed. Inhibition was established within less than 10 min.

3. Aerobic stimulation of glycolysis by the addition of potassium ions was equally rapid in onset. Its main development occurred following the addition of 30–50 m.equiv./l.

4. Diminution of the total salt concentration by an amount equivalent to the added potassium did not interfere with the effects of potassium ions on glycolysis.

I am very grateful to Prof. H. R. Dean for his kind interest in this work and to Dr G. D. Greville for most helpful discussion and advice. I also wish to thank Mr D. Madin for valuable technical assistance.

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SODIUM THIOSULPHATE EXCRETION IN THE CAT

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It has recently been claimed that thiosulphate is excreted in the same manner as inulin in man (Newman, Gilman & Philips, 1946), and as creatinine in the dog (Gilman, Philips & Koelle, 1946; Pitts & Lotspeich, 1947): in other words, that its clearance provides a true measure of glomerular filtration rate.

An investigation into the treatment of this substance by the kidney of the cat, over a wide range of variations in its concentration in the plasma, indicated that in this animal thiosulphate clearance does not provide a measure of glomerular filtration rate but that it is actively secreted by the tubule cells. This result confirms and amplifies the suggestion made by Bing & Effersøe (1948) while this work was in progress.

METHODS

All experiments were performed on cats under nembutal (pentobarbital sodium) anaesthesia. The bladder was cannulated through the urethra, the main bulk of the bladder being tied off from the trigone and entering ureters, to minimize the dead space. Infusions were made into one external jugular vein, arterial blood pressure recorded from a carotid artery, and blood samples taken either from a femoral artery when large enough, or from the other carotid artery.

Creatinine clearance was used as a measure of glomerular filtration rate (G.F.R.), since it is known to remain constant during short-term experiments, and to be independent of variations in plasma creatinine concentration (Eggleton, 1944). The use of inulin clearance for this purpose has been under investigation, but preliminary results indicate a behaviour so irregular in relation to the steadiness shown by creatinine clearance that the latter has been retained.

The experiments were of two main types. In a few, a first rapid infusion of creatinine and sodium thiosulphate (freshly prepared) was followed by a continuous slow infusion and, when urine flow was steady, two consecutive samples were collected, followed by a blood sample. In the majority of experiments, a single injection was made either of creatinine, of thiosulphate, or of both together; the first blood sample was taken 5–30 min. after the end of the infusion and at intervals thereafter, the clearance during the collection of the intervening urine samples being calculated from the concentration in the plasma at the mid-point of each sample. It is unorthodox to attempt sampling before equilibrium has been attained between blood and tissues, and we were first driven to this expedient when using small injections of thiosulphate, since this substance disappeared too rapidly

to allow reasonable sampling after the customary time lag. Experience showed, however, that determinations of clearance were quite as accurate in this early stage as after equilibrium had been attained, and the timing of the first blood sample was thereafter varied between 5 and 30 min. after the end of the injection as convenient in individual experiments. The fluid balance of the body was maintained by infusion of normal saline at a rate equal to that of the urine flow.

Thiosulphate was determined in plasma and urine by the methods of Gilman Philips & Koelle (1946), with one slight modification. The 10 and 25 c.c. iodate solution used in the analysis of plasma and urine respectively were reduced to 5 and 10 c.c., thus allowing the use of smaller volumes of plasma filtrate and urine. In many experiments a blank determination was made on both plasma and urine. The plasma value varied from 1·4 to 3·5 mg./100 c.c. (average of eight values was 2·7 mg./100 c.c.), but estimations of such low values are inaccurate. The urine blank value varied from 0·016 to 0·36 mg./min., the variation being partly individual and partly dependent on rate of urine flow, the blank increasing with increasing rate of flow. In view of these uncertainties, together with the fact that final results differed little whether the blank values were subtracted or not, the results as presented are uncorrected.

Creatinine was determined as the alkaline picrate, but difficulty was encountered in the presence of a high concentration of thiosulphate owing to the development of a turbidity in the picric acid solution, which disappeared only very slowly when the solution was made alkaline. The creatinine concentration in plasma, therefore, was determined in a neutral filtrate (tungstate), the picric acid and NaOH being added simultaneously, and the colour comparisons made within 15–30 min. after this addition (Hawk, Oser & Summerson, 1947). The method was found to be equally applicable to urine, the volume being made up to 10 c.c. with water before the addition of the sodium picrate.

Determination of plasma flow was made by constant infusion of 0·2–0·25 % *p*-aminohippuric acid (freshly neutralized) at 0·2–0·4 c.c./min., following an initial rapid injection (10–15 min.) of 25 c.c. of 0·1 % solution. In a few experiments a high concentration of this substance (PAH) in the plasma was attained by an initial injection of 25 c.c. of 1–2 % solution, followed by a steady infusion of 6–8 % at 0·2 c.c./min. PAH was determined by the method of Smith, Finkelstein, Aliminosa, Crawford & Graber (1945), with one slight modification. Formation of gas bubbles during the colour development proved troublesome and was overcome, without loss of accuracy in the analysis, by replacement of the 100 mg. nitrite/100 c.c. solution advocated with one of 20 mg./100 c.c.

Phosphate clearance was determined in several experiments, the value of the last plasma sample being used for comparison with all urine samples in each experiment. The method of Youngburg & Youngburg, as described by Hawk & Bergeim (1938), was used for its estimation both in plasma and urine.

RESULTS

Thiosulphate/creatinine clearance ratio. The results of an experiment showing the dependence of thiosulphate clearance on the concentration of this substance in the plasma are shown in Fig. 1. At a concentration of 40–20 mg./100 c.c., the clearance ratio rises from 1·6 to 1·8, in contrast with the value of 1·1–1·4, as the concentration falls from 180 to 70 mg./100 c.c. The results also illustrate the fact, established in many dozens of experiments, that creatinine clearance is completely independent of the concentration of this substance in the plasma. The clearance remains constant throughout the experiment in this particular instance and, in comparison with some later results, attention is called to the fact that the second injection of thiosulphate was given slowly. In this experiment, as in all others, the creatinine clearance is independent of changes in rate of urine flow. The latter is abnormally high for a cat owing to the diuretic action of thiosulphate.

In Fig. 2 the thiosulphate/creatinine clearance ratios from close on fifty observations in seven different cats have been plotted against thiosulphate concentration in the plasma. The ringed crosses denote values which were obtained after a second injection of thiosulphate, as in the experiment depicted in Fig. 1. The degree of scatter of individual points about the line which has been drawn through them (by eye) is no greater than is expected in experiments

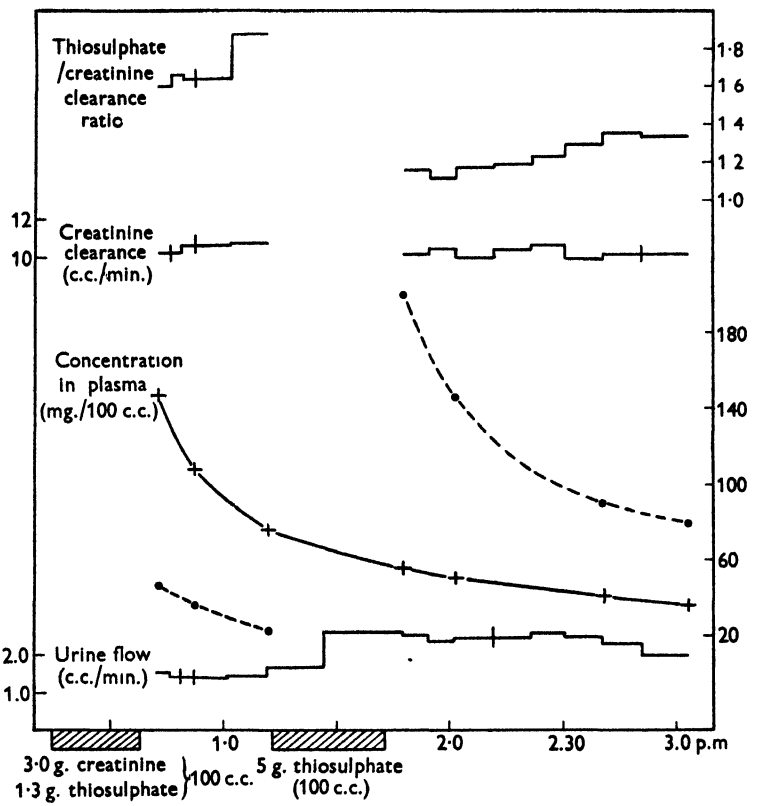

Fig. 1. The variation of thiosulphate clearance with changing concentration in the plasma. Cat, 7·25 kg. ×———×, Creatinine; •- - - - - -•, thiosulphate.

of this type and leaves no doubt that thiosulphate clearance increases progressively with decrease in concentration in the plasma. According to current theory, therefore, thiosulphate is actively secreted by the renal tubule cells.

Thiosulphate Tm. The actual amount secreted can be calculated from the total amount excreted and the amount filtered. This value was found to vary widely from one experiment to another, in the same direction as similarly wide variations in creatinine clearance (hereafter referred to as G.F.R.). Unlike normal dog and man, no relationship was found in this group of cats between G.F.R. and body weight or surface. Large animals were used inten-

tionally (3·5–7·75 kg.) to minimize the effect of frequent blood sampling, and it would seem that renal function is impaired with advancing years. A comparison of the results obtained on different animals was made, therefore, in terms of G.F.R. in place of body weight, and when the amount of thiosulphate secreted was expressed as mg./min./100 c.c. G.F.R., it was found to increase with increasing concentration of thiosulphate in the plasma up to a concentration of *c.* 40 mg./100 c.c. Further increase in concentration (up to 190 mg./100 c.c.) was accompanied by no further increase in the amount secreted. The results of one typical experiment are given in Table 1 and the results of seven

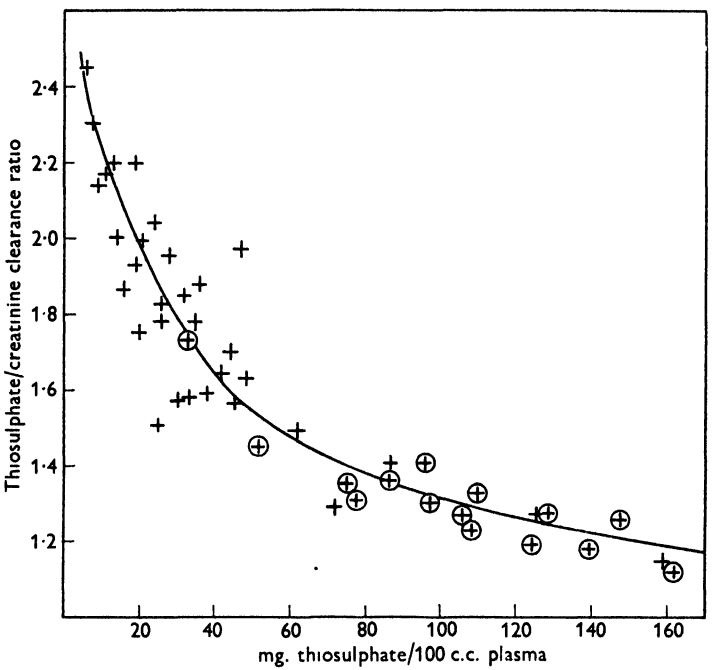

Fig. 2. The relationship between thiosulphate/creatinine clearance ratio and thiosulphate concentration in the plasma. ⊕ denotes values obtained after a second injection of thiosulphate.

are plotted in Fig. 3. The results of three others, of which mention will be made later, have been omitted from the figure. There is a considerable degree of scatter in the individual values which can be, in part, attributed to the large number of animals used, as compared with the one or two dogs normally used in work of this nature. The greater degree of scatter at the higher concentrations of thiosulphate in the plasma is to be expected, the effect of technical errors mounting as the amount secreted becomes a small difference between two large values. The average of four values obtained at a concentration in the plasma greater than 140 mg./100 c.c. was 29 mg./min./100 c.c. G.F.R. and the average of all twenty values at a concentration greater than 40 mg. thio-

sulphate/100 c.c. plasma was 28·3 mg./min./100 c.c. G.F.R. We may define the *Tm* value for thiosulphate secretion in the cat, therefore, as 25–30 mg./min./100 c.c. G.F.R.

TABLE 1. The secretion of thiosulphate in relation to its concentration in the plasma. Cat, 3·5 kg.

G.F.R. (c.c./min.)	Plasma thiosulphate (mg./100 c.c.)	Thiosulphate (mg./min.) Filtered	Excreted	Secreted	Secreted/100 c.c. G.F.R.
15	48·5	7·3	11·85	4·55	30·5
14·7	35	5·15	9·2	4·05	27·5
16	25·8	4·15	7·6	3·45	21·5
15	19	2·85	5·5	2·75	18·4
15·7	13·8	2·17	4·37	2·2	14·0

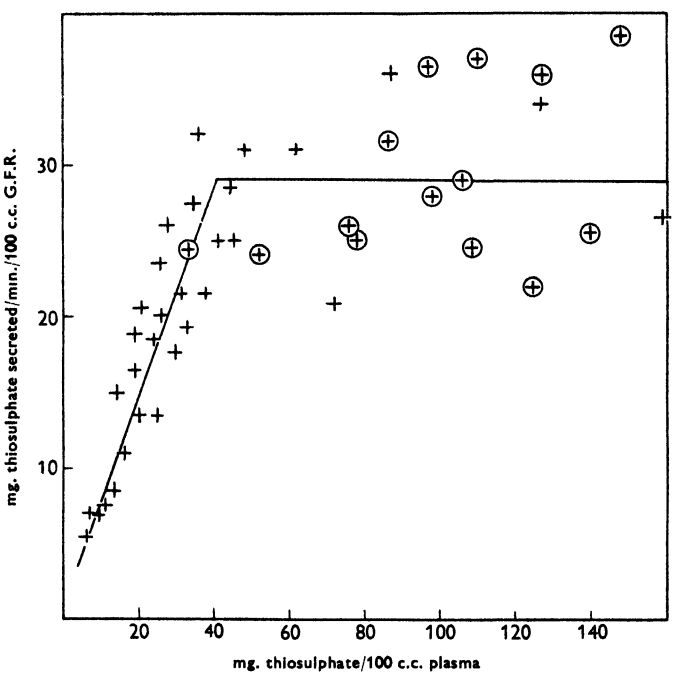

Fig. 3. The existence of a maximal secretion rate of thiosulphate by the kidney. ⊕ denotes values obtained after a second injection of thiosulphate.

In one experiment, whose results are omitted from the figure, six values were provided at a concentration ranging from 115 to 76 mg./100 c.c., yielding an average value of only 13 mg./min./100 c.c. G.F.R. A partial block occurred in the urethral cannula at an early stage, but was only detected half-way through the infusion of thiosulphate and creatinine. Thereafter, the urine flow was consistently low, in spite of the amount of thiosulphate injected (2·5 g.), blood was present in the urine throughout the experiment, the filtration rate was abnormally low (4–6 c.c./min.), and the kidneys examined at the end were very tense.

The abnormally low values obtained for the amount of thiosulphate secreted suggests that serious back pressure through the ureters, with consequent rise in intrarenal pressure for any

length of time (10–15 min. in this case) may cause more permanent damage to the tubule cells than to the glomeruli. The low G.F.R. is not itself the cause of such a reduction in tubular function, for in another large animal (7·75 kg., previously treated with anterior pituitary extract) in which there was no hint of a block, the G.F.R. was only 3–4 c.c./min. throughout, but the amount of thiosulphate secreted was within the normal range.

In a second experiment, whose results are omitted from the figure, 'leaky' tubules were undoubtedly responsible for an apparent slight reabsorption of thiosulphate. The cat (4 kg.) was obviously badly infected and had a brownish deposit in the urine, the initial creatinine clearance being only 2·7 c.c./min. Injection of thiosulphate resulted in the appearance of blood in the urine and a *rise* in creatinine clearance to 3·7 c.c./min., the thiosulphate clearance being only 3·2 c.c./min. (concentration in plasma 194 mg./100 c.c.). This rise in creatinine clearance was associated with an increase in rate of urine flow from 0·2 to 0·8 c.c./min., a relationship to be expected if creatinine were leaking back to the blood stream through the tubule cells. The apparent reabsorption of thiosulphate is equally well explained since thiosulphate is known to diffuse more rapidly than creatinine.

Depressant action of thiosulphate on the kidney. In the earlier experiments thiosulphate and creatinine were injected together, but in a later experiment, creatinine clearance was determined before any thiosulphate was given. Injection of the latter led to an appreciable and apparently permanent depression of G.F.R. The experiment was repeated several times, with various modifications, and invariably the same effect was observed. Further investigation showed that a second injection of thiosulphate could produce a further reduction, and in all cases the depression of G.F.R. appeared to depend as much, if not more, on the speed with which the injection was made as on the absolute amount given. The results of an experiment in which a drastic depression of G.F.R. was produced are shown in Fig. 4. Both injections were begun at high speed and slowed down only when the heart showed signs of over-strain. After the first injection, there is sign of some recovery, but little after the second. It is to be noted that this depression is not associated with changes in general arterial pressure, and that the thiosulphate/creatinine clearance ratio is relatively little affected.

Of the two possible causes of the depression in creatinine clearance—actual depression of G.F.R. or damaged tubules permitting a leaking back of creatinine to the blood stream—available evidence supports the former, and strongly suggests that filtration ceases altogether at many nephrons rather than suffering some reduction in all. This evidence comes from three different sources:

(1) A reduction in G.F.R. following a second injection of thiosulphate is not accompanied by any change in the thiosulphate/creatinine clearance ratio as can be seen in Fig. 2, i.e. the amount of thiosulphate secreted per 100 c.c. G.F.R. is unchanged (Fig. 3). Were the same number of nephrons functioning, with a reduced G.F.R. in each, the absolute amount of thosulphate secreted should be unchanged with a corresponding increase per 100 c.c. G.F.R. Had the tubule cells become damaged in any way, the amount of thiosulphate secreted might be less than normal, or the secretion normal but apparently reduced by a leak back

through the tubule cells. In one experiment only has there been any indication of tubular damage. An initial G.F.R. of 20 c.c./min. was reduced to 5 c.c./min. following a rapid injection of 3·8 g. thiosulphate. During the course of the next half an hour it rose to 8·5 c.c./min., the rate of thiosulphate secretion being 10 mg./min./100 c.c. G.F.R. in place of the normal 30 mg. Thereafter, the G.F.R. showed no appreciable change, but thiosulphate secretion had risen to 18 mg./min./100 c.c. G.F.R. after a further half an hour.

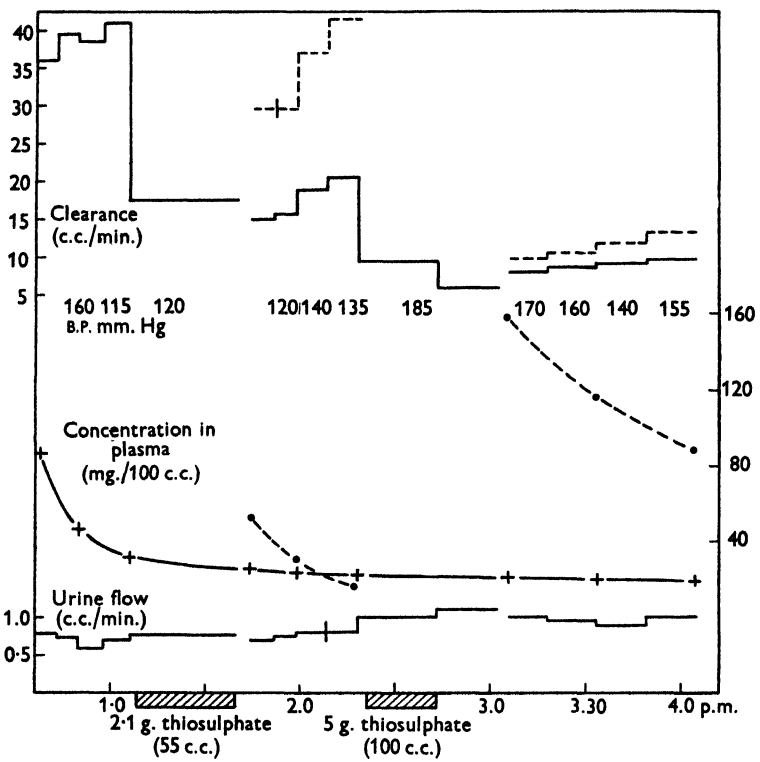

Fig. 4. The depressive action of two successive injections of thiosulphate on the creatinine clearance. Cat, 6 kg. ×———— ×, creatinine; •- - - - - -•, thiosulphate. The creatinine injection (2·5 g. in 70 c.c.) was given from 12.12 to 12.32 p.m.

(2) In the four experiments in which plasma flow has been determined by PAH clearance, this clearance has fallen to nearly the same extent as G.F.R. The results of an experiment given in Table 2 show the closeness of this relationship. The PAH clearance is not reduced to quite the same extent as is the G.F.R., suggesting that the blood flow is somewhat increased in those nephrons which continue to function. Had the depressant action of thiosulphate been primarily on tubular function, one would have expected a greater depression in PAH clearance than in that of creatinine, since so much of the former is actively secreted.

TABLE 2. The effect of thiosulphate on G.F.R. and renal plasma flow. Cat, 3·5 kg.

Concentration in plasma (mg./100 c.c.)		Clearance (c.c./min.)			
Creatinine	PAH	Creatinine	PAH	PAH/creatinine clearance ratio	Blood pressure (mm. Hg)
173	2·01	19·5	57	2·9	150
111	1·53	19·8	57	2·9	140
		20·7	62	3·0	125
69	1·23	19·6	62·5	3·2	115
100 c.c. 3·8% thiosulphate injected intravenously					
		7·1	28·5	4·0	125
61·3	1·89	3·15	18	5·7	155
		5·45	25	4·6	165
56·6	2·52	7·0	30·5	4·35	165
		8·1	33	4·1	155
50	2·52	8·5	33·2	3·9	150
		8·95	36	4·0	130
39·2	2·22	8·8	36·2	4·1	135

(3) In six experiments, phosphate clearance has been determined and the amount reabsorbed calculated. Whatever the degree of depression of G.F.R. caused by injection of thiosulphate, the amount of phosphate reabsorbed/100 c.c. G.F.R. has remained unchanged. The results of an experiment given in Table 3

TABLE 3. The effect of thiosulphate on reabsorption of phosphate. Cat, 6 kg. Plasma *P* 4·5 mg./100 c.c.

G.F.R. (c.c./min.)	Phosphate (mg. *P*/min.)				Phosphate/ creatinine clearance ratio
	Filtered	Excreted	Reabsorbed	Reabsorded/ 100 c.c. G.F.R.	
39·5	1·78	0·49	1·29	3·25	0·275
41	1·84	0·445	1·395	3·4	0·24
2·1 g. thiosulphate (55 c.c.) injected intravenously					
15·8	0·71	0·175	0·535	3·4	0·245
5·0 g. thiosulphate (100 c.c.) injected intravenously					
6·0	0·27	0·11	0·16	2·67	0·4
9·4	0·422	0·2	0·222	2·38	0·47

are typical of all. Phosphate clearance falls as G.F.R. falls and the absolute amount reabsorbed diminishes in proportion, leaving the amount reabsorbed per 100 c.c. G.F.R. practically constant, at 2·4–3·4 mg./min./100 c.c. G.F.R. Had the phosphate concentration in the plasma been determined early in the experiment, the results would have been even more consistent, for later experience has shown that this concentration tends to rise when G.F.R. has been reduced. It is difficult to conceive of any explanation of the constancy of this value following thiosulphate injection other than a reduction in the number of functioning nephrons.

Effect of p-*aminohippuric acid on thiosulphate secretion.* In three experiments, not included in the foregoing account, thiosulphate was injected at a time when a high PAH concentration was being maintained in the blood plasma. Eleven values were obtained for the amount of thiosulphate secreted when its

concentration in the plasma was greater than 40 mg./100 c.c. In place of the normal 25–30 mg./min./100 c.c. G.F.R., these values ranged from 0 to 13, with an average value of 10 mg./min./100 c.c. G.F.R. Thus, PAH in high concentration (37–138 mg./100 c.c.) depresses the secretion of thiosulphate as it has been shown to depress that of various other substances (Pitts, 1946). Thiosulphate clearance is also depressed by PAH in man (Crawford, 1948), but the action here is attributed to disturbances in the glomerular circulation, since inulin clearance is depressed to the same extent. This matter will be discussed in greater detail in a later publication.

DISCUSSION

The investigation was undertaken in the hope and expectation that thiosulphate clearance would provide a measure of G.F.R. in the cat, as it has apparently been shown to do in man and the dog. In view of the unexpected results obtained, the previous work was scrutinized more critically, for with the large error inherent in all experiments of this type, the relationship now established in the cat might well be missed unless the effect of a wide range of thiosulphate concentration in the plasma was investigated. This is not easy, for large doses of the substance produc etoxic effects: on the kidney, as we have observed; and causing nausea and vomiting in man, dog and cat (noted in the cat both by Bing & Effersøe (1948) and by ourselves). Moreover, small concentrations in the plasma cannot be determined accurately unless a large volume (up to 3 c.c.) of plasma is used.

In the one publication of work on man (Newman Gilman & Philips, 1946), the authors find a range of thiosulphate/inulin clearance ratios of 0·8–1·2, and attribute this scatter to experimental error. The range of thiosulphate concentration in the plasma was 10–60 mg./100 c.c. However, in the one experiment of which details are given, the ratio starts at 0·94 and rises steadily and consistently to 1·1 as the concentration in the plasma falls from 40 to 10 mg./100 c.c. It seems not improbable that had all the experiments been analysed in this way, the large scatter of 0·8–1·2 in the clearance ratio observed could be attributed, not to experimental error, but to a relationship with the thiosulphate concentration in the plasma. Should this prove correct, further investigation would seem essential to establish the actual range of clearance ratio, for the results as they stand indicate secretion of thiosulphate at low and reabsorption at high concentrations, a rather unusual state of affairs.

The original investigation of Gilman *et al.* (1946) was carried out on five dogs, mostly under pentobarbital anaesthesia. In nine experiments on three of these, there is no hint of any increase in thiosulphate clearance with decreasing concentration in the plasma, and in one experiment there is a slight decrease. In all four experiments on the other two animals, there is a pronounced increase: from 66 to 91, 49 to 73, 58 to 75 and 58 to 80 respectively. In only

two of these, however, is there a consistent increase in the thiosulphate/creatinine clearance ratio and that of much smaller magnitude; in other words, the creatinine clearance also apparently rose steadily throughout these four experiments. The authors make no comment on these increases, but elsewhere say 'one is tempted to attribute the variations (in clearance ratio) to the inherent errors in the colorimetric determination of creatinine'. In the later work of Pitts & Lotspeich (1947) on the dog, the effect of changes in thiosulphate concentration in the plasma on clearance ratio was studied in twelve experiments, the concentration ranging from 18 to 55 mg./100 c.c.; there was no consistent change in the clearance ratio. Thus it would seem that thiosulphate clearance is a measure of G.F.R. in the majority, possibly of all, dogs, though the evidence suggests that in some dogs active secretion may occur, as in the cat.

The depression in creatinine clearance caused by thiosulphate was not noted by Bing & Effersøe (1948). The two substances were always injected together in their experiments. Their range of creatinine clearance is given as 0·17–17·5 c.c./min. in eleven cats of 2–2·5 kg. weight, values much lower than those found by us in normal cats in this country. In a recent series of larger animals, an average value of 20·5 c.c./min. was observed; and in an earlier series of forty-seven smaller animals (1·5–4·7 kg.) the creatinine clearance ranged from 3–45 c.c./min., with an average of 15. The low values obtained by Bing & Effersøe can, therefore, reasonably be attributed to the depressant action of thiosulphate.

This depressant action appears to be mainly due to complete cessation of function in some nephrons, the remainder retaining both normal G.F.R. and tubular function, except *in extremis*, for reabsorption of phosphate and secretion of both thiosulphate and PAH diminish in proportion to the fall in creatinine clearance. This should not occur if the tubules of all nephrons were functioning with a diminished flow of plasma filtrate in each. The alternative hypothesis, that creatinine clearance is reduced by thiosulphate because the tubules have become 'leaky', involves the consequence that creatinine, thiosulphate, PAH and phosphate all diffuse back through the tubule cells at the same rate—a most unlikely event. The depression lasts for some hours, but its permanency beyond this period has not been studied.

Thiosulphate has been used clinically for many purposes, and although it is realized that the solution must be freshly prepared and that the injection must not be too rapid if nausea and vomiting are to be avoided, the actual dosage frequently used is such that the possibility of a depressant action on renal function cannot be ignored. For example, Litwins, Boyd & Greenwald (1943) recommend the use of 50 c.c. of a 50% solution intravenously to prolong the blood-clotting time in cases of post-operative thrombophlebitis. They advocate a slow infusion, but do not specify the rate. This dose of 25 g. is nearly double

that advised by Newman *et al.* (1946) as the safe limit in their studies on renal function in man; even with this dose, the latter authors note that 'preliminary observations show that injections of thiosulphate cause no change in inulin and in PAH clearance but more studies are necessary to establish this beyond question'. The results now obtained on cats add force to this suggestion that more studies are necessary, especially in view of the higher doses used in clinical work.

Attention was drawn by the earlier workers to the uniqueness of thiosulphate amongst anions in failing to be reabsorbed by the tubule cells and the present demonstration of its active secretion adds to this uniqueness. The findings of Newman *et al.* (1946) in man, to which reference has already been made, could be explained as they stand on the hypothesis that thiosulphate shares with other anions some tendency to be reabsorbed, but that there exists also a separate secretory mechanism. The few experiments of Gilman *et al.* (1946) on the dog also are not incompatible with this view. Their lowest clearance ratio of 0·92 was obtained at a high concentration of thiosulphate (150 mg./100 c.c.), and their highest of 1·2 at a concentration of 20 mg./100 c.c. In the cat, a clearance ratio of less than 1·0 has not been observed, except in the one diseased animal already noted, and if reabsorption of thiosulphate does occur, it must be very much smaller in relation to the amount secreted than in man or the dog.

SUMMARY

1. Sodium thiosulphate is actively secreted by the renal tubule cells in the cat's kidney, the thiosulphate/creatinine clearance ratio rising with falling thiosulphate concentration in the plasma (Figs. 1 and 2).

2. The maximal rate of secretion is 25–30 mg./min./100 c.c. G.F.R. and is reached at a plasma concentration of 40–50 mg./100 c.c. (Fig. 3 and Table 1).

3. This maximal rate is seriously reduced by the presence of a high concentration of PAH in the plasma.

4. Thiosulphate exerts a depressing action on the kidney, reducing both glomerular and tubular function to the same extent: the rate of reabsorption of phosphate and of secretion of thiosulphate and PAH fall with a fall in the creatinine clearance (Tables 2 and 3).

5. The degree of depression so produced appears to vary in individual animals and can be correlated to some extent with the speed of injection of thiosulphate as well as with the absolute amount given.

Our warm thanks are due to Dr K. H. Beyer for a supply of PAH from Messrs Sharp and Dohme Inc. when it was unavailable elsewhere.

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THE RELATION OF STRUCTURE TO THE SPREAD OF EXCITATION IN THE FROG'S SCIATIC TRUNK

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In the past a great many attempts have been made to explain quantitatively the spread of current and of excitability about electrodes on a frog's nerve trunk in terms of the electrical properties of a simple cable consisting of a resistant (and capacitative) cylindrical membrane separating a conducting core from a conducting outside medium. We have shown (Rashbass & Rushton, 1949*b*) that in at least three important respects the excitability of the sciatic trunk does not behave as it would if this were so. For the excitation does not always arise at the cathode, the excitability does not fall away exponentially on either side of a single electrode, nor are the results the same when the nerve is stimulated by bipolar electrodes as by the symmetrical tripolar arrangement. In the present paper we attempt to show how these results can be explained in terms of the electrical properties of the structures of the nerve trunk.

In 1926 Bishop, Erlanger & Gasser drew attention to the appreciable electrical resistance (and capacity) of the nerve's connective tissue sheath, and suggested that 'many of the properties of the nerve as usually measured may be in fact the property of this non-nervous structure'. Although since then various workers (see Discussion) have given differing significance to the part played by the epineurium, its contribution to the spatial characteristics of nerve has not been extensively investigated. In the first part of this paper we show that when the epineurial connective sheath has been removed, the nerve follows closely the expectations of the simple cable theory, and in the second part we show by what property the connective tissue brings about those deviations from the simple theory which are exhibited by the unstripped nerve.

Part 1. THE SPREAD OF EXCITATION IN STRIPPED NERVE

Methods

Dissection. The sciatic nerve of the frog was dissected out from the cord to the end of the peroneal branch, the tibial branch being ligatured and cut short, but the branches to the hamstrings left rather long. The nerve was placed on the stage of a dissecting microscope and illuminated

from above. The ligatures on the central and the cut tibial branch were held down by clips, keeping the main trunk under a slight degree of tension. The removal of the epineurium, performed under the microscope, was carried out entirely by means of two steel needles with sharp points. These were used to cut the sheath either by compressing it downwards against the microscope stage, or outwards one needle against the other. The first incision of the sheath was made in the fork between the hamstring branch and the main trunk. The cut was extended right round the trunk, dividing the sheath in two, and then a longitudinal slit was made down to the bifurcation at knee level. The epineurium could then be removed from 15 to 20 mm. of the thigh region.

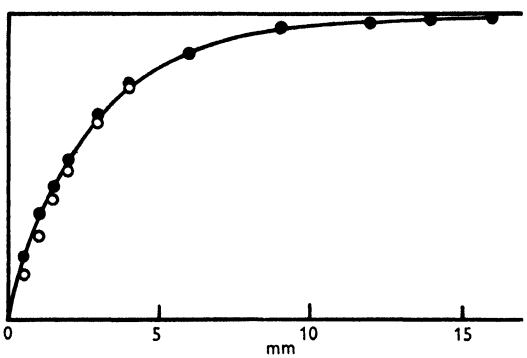

Fig. 1. Excitability curve of stripped nerve. Abscissae: interpolar length. Ordinates: excitability (=reciprocal of current strength for ¼ maximal response).

Experiment. The nerve, prepared in this fashion, was embedded from knee level upwards in a cylinder of agar jelly, and set up in the apparatus which has been previously described (Rashbass & Rushton, 1949*b*). In the majority of experiments where the stripping and embedding of the nerve were accomplished without mishap, these operations resulted in no appreciable reduction of the maximum action potential recorded from the peroneal branch. For this and other reasons to be mentioned later, we believe the nerve to be quite healthy despite these manipulations. The rest of the experimental procedure, which was the same as in our previous experiments on unstripped nerves, consisted in measuring the threshold for bipolar and symmetrical tripolar stimulation with various electrode separations.

Results

It will be recalled that the simple cable theory requires that the excitability falls away on either side of a single pole according to the exponential curve $e^{-x/\lambda}$, where x is the absolute distance away from the pole and λ is the space constant. Thus excitation will always occur at the cathode, and by the Superposition Theorem, bipolar and symmetrical tripolar thresholds will be the same (Rashbass & Rushton, 1949*b*). If then the excitability curves are scaled to have a maximum value 1, they should both coincide with the exponential $1-e^{-x/\lambda}$. Now the results of one experiment upon a large Swiss variety of *Rana temporaria* are plotted in Fig. 1. Dots show the bipolar excitability; circles, the symmetrical tripolar excitability. The curve $1-e^{-x/\lambda}$ is also drawn where $\lambda=2{\cdot}8$ mm., chosen to give the best fit. The curve fits the bipolar excitability closely, but the tripolar points are seen to lie below the curve for interpolar distances less than 4 mm., though the divergence is very small when

compared with the corresponding curves (Fig. 2) taken from the same nerve before removal of the epineurium (see Part 2). Latency measurements, in the experiments in which they were determined, showed that at small electrode separations, the point of excitation was still displaced from the cathode, but to a rather less extent than when the connective tissue sheath was intact. These results indicate, therefore, that the nerve, after removing the epineurium, behaves very much more like a simple cable than before.

There are several factors which may contribute to the discrepancies which remain. (*a*) The connective tissue is never entirely removed. However efficiently the main sheath is dissected away, there always remain septa of connective tissue which pass among the nerve fibres in the trunk, subdividing it into smaller bundles. These bundles Tasaki (1939*a*) found to affect current distribution, and they may, in part at least, contribute to the slight divergence

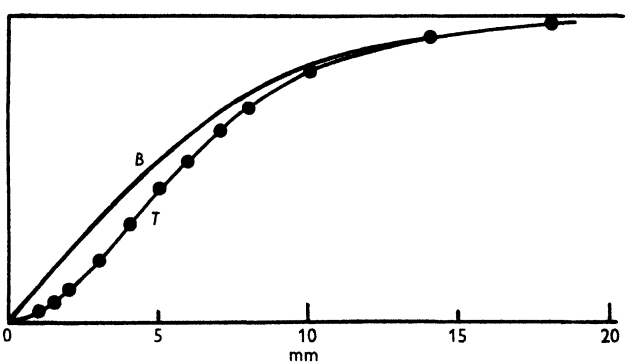

Fig. 2. Excitability of the nerve before stripping plotted as in Fig. 1. *B*, bipolar stimulation; *T*, symmetrical tripolar stimulation.

between the two excitabilities of Fig. 1. (*b*) Though the change in potential gradient at the electrode is sharp (Fig. 2, Rashbass & Rushton, 1949*b*) it is not perfect, so that the tripolar stimulus is physically not precisely the superposition of two dipolar ones. (*c*) The fibres in the centre of the nerve are surrounded by others, whose effect would be to enhance (*a*). We believe this contribution to be very small (see end of Part 2). (*d*) Probably the most important and certainly the most interesting consideration arises from the fact that fibres are only excitable at the nodes of Ranvier (Kato, 1934, 1936; Tasaki, 1939*a*, *b*). It is thus clearly insufficient to consider simply at which point in a cable the maximum depolarization occurs, we must consider which *node* is most depolarized. This distinction is not important in unstripped nerve since here the excitability alters only gradually with distance. But in the experiment of Fig. 1, we should certainly expect that the excitability relations of each nerve fibre would depend significantly upon the proximity of its nearest node to the cathode. Moreover, the variation among fibres in this respect

will account qualitatively for the observed deviation from the simple cable expectations.

For, in bipolar excitation with the anode close to the cathode, though the excitability will be maximal exactly at the cathode, it will fall away much faster towards the anode than away from it, consequently the majority of the nodes will be excited in the region just extrapolar to the cathode. This will account for the observed latency shift, and the inexactness of comparing bipolar and tripolar excitations by superposition. But the tripolar conclusions are more striking. As the two anodes approach the cathode on either side, they diminish the cathodal stretch of nerve, and sharpen the peak of excitability. Distant nodes become relatively less easily excited, and eventually are excluded from the cathodal stretch entirely. A proportion of the nerve fibres will in this way become inexcitable. Thus three conclusions follow when the anodes are close. (i) The tripolar curve will lie below the bipolar (Fig. 1). (ii) The divergence will be more, the greater the size of action potential selected as index of excitation. (iii) There will be a reduction in the maximal action potential obtainable owing to the exclusion of some fibres.

These three phenomena have been regularly observed. But clearly many factors besides the distribution of nodes may enter into their interpretation.

Asymmetrical tripolar stimulation

Tasaki (1939*b*) has stimulated a single nerve fibre through three electrodes X, O, Y applied to consecutive nodes. Simultaneous current pulses through X and Y could be independently adjusted with regard to strength and direction, the sum of the currents returning through O. For arbitrary values of the current x (through X) the value of y (through Y) was found which was just threshold. Then y plotted against x in the usual way resulted in a triangle, each straight line corresponding to excitation at one of the three nodes. In our previous paper (1949*b*) we performed the same experiment upon the whole (unstripped) nerve, and, for interelectrode distances of 1 or 2 mm., got results quite different from Tasaki's.

It now seems natural to explain the difference in terms of the connective tissue sheath, for we have seen in Figs. 1 and 2 above how by stripping, we change the symmetrical tripolar curve very nearly to the expectations of the simple cable theory. Moreover, we have shown that the Superposition Theorem, applied to the curve of Fig. 2, will accurately predict our non-triangular results with unstripped nerve, and Rushton (1928) proved that the simple cable theory always predicts a triangle, such as was found by Tasaki.

We have therefore repeated upon stripped nerve the experiments of the previous paper, and Fig. 3 shows the results upon the same nerve which yielded Figs. 1 and 2, the interelectrode distance $XO = OY$ being 1, 2, 3, or 4 mm. As before the observed results are plotted above the 45° line ($x = y$);

below it is plotted the expectation from the simple cable theory when $\lambda = 2{\cdot}8$ mm., as found in Fig. 1. The shape of these triangles therefore is completely determined. If observations fitted this theory exactly, the points above the line would be precisely a reflexion of the curve below. In fact, there are two kinds of deviation: (*a*) In the first quadrant (where both X and Y are

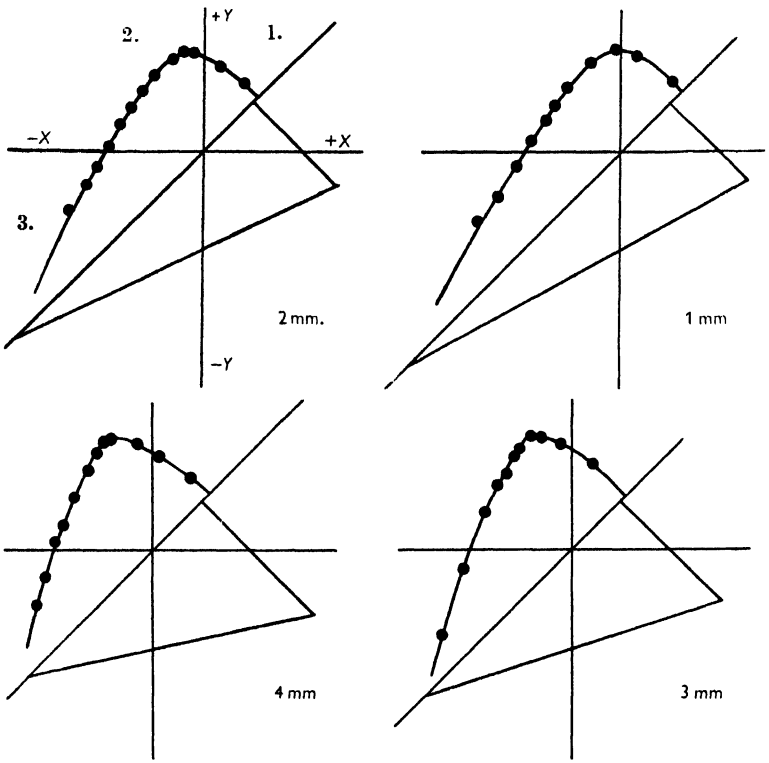

Fig. 3. Results of stimulating the stripped nerve with tripolar electrodes with distance $OX = OY = 1$, 2, 3, 4 mm. respectively. Current through X plotted against Y for threshold. Only the experimental points above the 45° line are shown. Below are the theoretical results calculated from the simple cable theory. Upon the 2 mm. curve the axes and quadrants are labelled according to the usual convention.

anodes) the threshold is higher than expected. This we have already related to the distribution of nodes about the cathode, for it is the case of anodes close on either side of the cathode. (*b*) In the second quadrant, the corner of the triangle is rounded off, and the threshold is lower than expected. In theory this corner is where excitation arises simultaneously from electrodes O and X. When O and X are close together, therefore, this region will correspond to a specially long cathodal stretch of nerve, and the decline of excitability from the two peaks at O and X will be more gradual than usual. Thus the same

factors in node distribution which raise the threshold in region (*a*), lower it in region (*b*). Residual connective tissue, of course, will equally account for the deviations from the simple cable results.

Anodal effects in stripped nerve

Anodal effects do not concern the main purpose of this paper so long as we are satisfied that the excitability concerned is the cathodal 'make' and not the anodal 'break', and also that no impulse arising at the cathode fails to reach the recording leads on account of anodal block. Both these effects are negligible in the ordinary way, but they are greatly enhanced by stripping the nerve. As

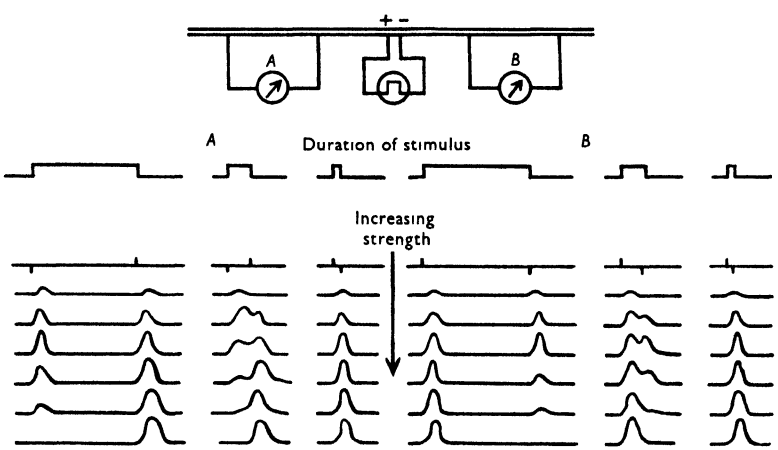

Fig. 4. Diagrammatic representation of stimulation and conduction block at the anode in stripped nerve, for currents of various strengths and durations.

the phenomenon is in itself interesting and possibly important, and since it certainly may affect the technique and interpretation of experiments with stripped nerve, we shall digress somewhat to discuss it.

Fig. 4 represents the matter diagrammatically. The left half of the figure indicates the results when the anode lies between the cathode and the recording leads; the right half, when it does not. Each half contains three columns of records corresponding to stimuli of, say, 10, 2, 0·5 msec. duration. Each column contains in diagrammatic form the action potentials seen with stimuli of various strengths. Consider first the results with 10 msec. duration of stimulus. In this case the action potential at 'make' is easily distinguished from that at 'break' because the latter arrives 10 msec. later on the cathode ray traverse. Moreover, if the instant of 'make' remains fixed on the traverse, and the duration of the stimulus is altered, the first or 'make' wave remains fixed, but the second wave moves with a constant latency behind the instant of

'break'. It will be seen from Fig. 4 that the thresholds for 'make' and 'break' are about equal, and the two waves increase to maximal in much the same way with increasing strength. But if the record is taken on the anodal side (left) the 'make' wave begins to decline soon after the maximal has been reached, and indeed sometimes before it, expecially when the electrodes are placed close together. A somewhat greater stimulus will reduce the response to zero. At this stage, by taking the record from the other end of the nerve or by reversing the polarity of the stimulus, we still obtain a full-sized action potential. So it is plain that the nerve has been maximally excited (for *A* fibres), and that all these have been blocked at the anode. The unusual susceptability to anodal block with stripped nerve is mentioned by Tasaki (1939*a*). Precisely the same thing happens in the reverse direction with anodal 'break' excitation. Here the interposition of the pole which had been the cathode produces a block which is altogether similar to the anodal block just considered. It is likely that some polarization of the thin silver-silver chloride electrodes contributed to the 'break' effects, but such polarization would of course apply equally to unstripped nerve which does not behave in this way.

As the duration of the stimulus is decreased, the threshold for 'break' rises faster than that for 'make' (as is well known); otherwise the results are the same. But it will be appreciated that when the duration of the stimulus is reduced to 0·5 msec. it is by no means obvious whether the excitation observed is cathodal 'make', or anodal 'break'. It is possible, however, to make certain of this by several methods. Latency measurements will decide between anode and cathode when the electrodes are well separated. The triangular results of Fig. 3 distinguished clearly which is the stimulating electrode and whether at 'make' or 'break' (for the isosceles triangle for 'break' is the other way up, with apex in the first quadrant and base in the third). Finally we may gradually increase the duration of the stimulus until the make and break waves are separate. It is usually easy to see that the wave to be analysed shows a smooth transition to one and not to the other of the final pair of waves.

We have applied such tests to the measurements described in the earlier part of this paper, and we are satisfied that all the thresholds discussed there are 'make' thresholds. Anodal block, too (with one possible exception) played no part. For only in symmetrical tripolar excitation was it necessary to place the anode in the path of conduction, and here it was found that the half-anode did not usually diminish the maximal action potential until the stimulus was raised considerably above maximal. Hence there was certainly no blocking at ½ maximal. The exception was when the two anodes were close on each side of the cathode. We have already spoken of the anomally of this case, which deviates from the simple cable theory, with a fall in the maximum response obtainable. This phenomenon could be due to the distribution of the nodes, it could be due to anodal block and there could be yet other factors.

In seeking a cause for the low threshold at which these classical anodal effects appeared, we considered three possibilities. (*a*) The nerve is in bad condition. (*b*) Our Ringer has a composition different from that of the interstitial fluid. (*c*) The effect is due in some way to the sharp change of excitability about the anode in stripped nerve. We were unable to obtain evidence in support of any of these suggestions in a variety of exploratory experiments.

(*a*) In support of the idea of a poor condition of nerve is the suspicion one naturally has of sheath-strippers however careful they say they are, and in fact our phenomenon did seem to increase a little with time. Against this are the following observations: (i) the maximum action potential (recorded from the unstripped peroneal nerve) was not reduced by stripping the sciatic nerve for a distance of 2 cm. about the excitation site; (ii) a stripped nerve left overnight in Ringer at room temperature, was found to be excitable next morning; (iii) stripping the nerve neither increased the refractory period nor diminished the maximum frequency of a tetanus which the nerve could conduct.

(*b*) The unstripped nerve is bathed by interstitial fluid; our phenomenon might be due to the change in this environment. It clearly was not due to embedding in agar jelly, for the results occurred equally when the stripped nerve was stimulated in Ringer or air. Against environmental significance are the following observations: (i) however long an excised unstripped nerve is allowed to equilibrate with Ringer, it never shows these properties; (ii) we were unable by applying fluids of different ionic compositions to restore a stripped nerve to 'normal' excitability.

We have not considered non-diffusible constituents.

(*c*) This paper leads to a precise idea of how removal of the epineurium changes the space distribution of current and excitability in nerve at anode and cathode, but the enhancement of anodal effects does not seem to follow as a logical consequence. Whether it follows as a physiological consequence could be established with certainty by applying to stripped nerve the external potential distribution which normally occurs in the interstitial region (see Part 2). We have not done this accurately, and rough approximations have given inconclusive results.

Conclusions to Part 1

Our former experiments upon unstripped nerve showed that the results would not fit the simple cable theory. Now we see that by removing the epineurium, we remove these discrepancies nearly entirely. Such as remain could well be due to residual connective tissue and the nodal structure of the nerves.

The cable to which the nerve approximates has a space constant $\lambda = 2{\cdot}8$ mm. (Fig. 1). This value was obtained in an experiment where the nerve resistance was very high compared with that of the surrounding agar-Ringer, hence 2·8 mm. is the 'characteristic length' of the most excitable fibres. This means that (conductivity of sheath per mm.) × (resistance of core per mm.)

$$= 1/(2{\cdot}8)^2 = 0{\cdot}13 \text{ mm.}^{-2}.$$

This value is about the average of our results with Swiss frogs. The English frogs (which were smaller and more emaciated) gave values of λ between 2·0 and 2·5 mm.

Part 2

In this paper our prime object is to study why the unstripped nerve exhibits a distribution of excitability so different from the expectations of the simple cable theory. In Part 1 we have seen that removal of the connective tissue sheath also removes most of the deviation from the simple cable theory. This proves that *in situ* most of the deviations were due to the presence of the epineurium, and the question now arises as to the nature of the epineurial influence. It is conceivable that the action is simply by holding the fibres close together as in a tight net, or some degree of impermeability may preserve a chemical environment for the nerve fibres different from Ringer's fluid. But by far the most likely suggestion is that the epineurium is somewhat resistant to the current which must flow across it when the nerve fibres within are excited.

This may be tested by inserting an electric probe into the interstitial fluid beneath the epineurium. For, if the sheath has electrical resistance, some fraction of the applied potential will be recorded across it, but if the resistance is negligible, as maintained by Lorente de Nó (1947*a*, *b*), so will be the recorded potential difference. As it turns out, the potential drop across the epineurium is far from negligible, but we have a much more stringent test than this to satisfy if we are to be assured that the epineurial resistance is not merely one factor but *the* factor which accounts for the deviation from the simple cable theory. For there is only one distribution of interstitial potential which will explain the observed excitability results, and unless we find that the interstitial fluid has this theoretical value of the potential at every point, it cannot afford a complete explanation.

The experiment, then, consists in essence of three kinds of measurements: (*a*) The spread of excitability about the cathode. This is best obtained by the symmetrical tripolar curve (Fig. 2). (*b*) The potential of the interstitial fluid beneath the epineurium, recorded directly from an inserted wire. Here it is best to apply the current through a pair of electrodes 1 mm. apart, so that the greatest possible fraction of the applied potential contributes to our measurement. (*c*) The nerve is now stripped and λ is measured as already described in Part 1.

Method

The whole set of experiments was always made upon the same nerve. The accuracy required is very high, and could only be achieved by a good deal of attention to detail. The best experiments were made upon large Swiss frogs (*R. temp*), whose sciatic-peroneal nerves were dissected out and set in a jelly rod as described in our previous paper (1949*b*), the epineurium remaining intact. The nerve was set up in the usual stimulating apparatus, and bipolar and symmetrical tripolar excitability curves obtained. The nerve was now removed from the apparatus, and the agar was gently cleaned away (which was not difficult) preparatory to a wire being introduced beneath the epineurium. The wire was of fine enamelled silver s.w.g. 42. One end was scraped bare for connexion to the amplifier, the other cut sharply across and insulated with a minute smear of 'durofix'.

Some 5 mm. from this tip the insulation was scraped away for a distance of 0·5 mm., and immediately before insertion into the nerve, the following insulation test was carried out.

A glass tube filled with Ringer's fluid had an electrode at each end so that a current pulse might be sent through the fluid. The middle of the tube was perforated to admit an Ag.AgCl recording electrode connected via a cathode follower to one input of the amplifier. The other input also via a cathode follower was connected to the fine wire to be tested whose bare silver surface, 5 mm. from the tip, was also chlorided. This wire was gently passed down the tube until the applied current pulse gave zero output. Usually this condition was when the bare area of the wire was exactly level with the other recording electrode, and in this case the wire was accepted (though the justification is not conclusive). Otherwise the specimen of wire was rejected.

It is important not to use the bare tip of the wire rather than the more complicated method just described, for in early experiments where this was done, the recorded potentials proved quite unreliable in many cases. We believe that this was because the sharp tip tended to pierce the epineurium either during the insertion or in subsequent manipulation, and even a partial entry would give a misleading result of the kind which we obtained. The more complicated method has the double advantage that the bead of 'durofix' over the tip removes its sharpness, and the potential is measured from a place which is usually well in the body of the nerve.

The insertion was performed under a dissecting microscope. A hole was torn in the epineurium of the tibial branch by a sharp needle, and the wire, held in forceps covered with soft material to protect the enamel, was pushed in and passed up the nerve within the sheath. The nerve rested on a mirror so the wire could always be seen through the epineurium either directly or reflected from below. Slight bending of the nerve helped to guide the wire tip more or less along the centre of the trunk. The wire was passed up until the bare patch was seen to lie about the middle of the branch-free thigh region. It was tied in this position by a ligature round the tibial branch near the point of entry of the wire.

The nerve with interstitial electrode was now again set in jelly, and the former experiment precisely repeated. This was to observe how far the normal excitability curve was changed by the insertion of the wire, its continued presence, and the other manipulations described. The change was usually small, and when it was not, the experiment was rejected. The nerve in its jelly rod is now carefully removed, and the apparatus prepared for measuring the interstitial potential.

The arrangement here is to send the current between two electrodes fixed at about 1 mm. apart, and to move the nerve along so that the bare part of the wire is at various distances from the electrodes. The nerve was attached at either end by Ag.AgCl hooks to an insulating framework held in a rack and pinion so that the jelly rod could be displaced as required correct to 0·1 mm. Unfortunately, two electrodes close together are very sensitive to irregularities both of moisture and contact as the jelly rod moves across them, and at first readings were quite unreliable. The difficulty was overcome by two procedures:

(*a*) Good contact was secured by casting for the nerve rod a jelly tunnel set around the electrodes. This was done before the nerve was moved into position. A glass rod 1 mm. in diameter was threaded through the wire loops which formed the electrodes, and a drop of hot agar-Ringer placed over the loops and rod. After setting, the rod was carefully withdrawn and the portions of jelly extrapolar to the electrodes cut away. Within this little agar tunnel the moist agar rod slid easily and with good fit, and the contact was fairly uniform as judged by passing a fixed current pulse through the electrodes in the jelly, and measuring the potential difference appearing between the two silver hooks supporting the ends of the nerve.

(*b*) The error remaining was reduced as a result of the observation that, though a change in moisture at the electrodes could still alter somewhat the potential difference both between the two hooks, and also between the internal wire and one of them, it changed these in nearly the same proportion. So it was only necessary to express the interstitial potential as a percentage of the potential difference between the hooks, to obtain a figure almost independent of irregularities in contact. In this way we believe that the readings of the interstitial potential were exact to about 1% of the potential difference of the stimulating electrodes. Readings were always repeated in

the reverse sequence, and the whole set was rejected unless every reading had been repeated within a tolerance of 2%.

Measurements were made as follows. The interstitial electrode was led to the grid of a cathode follower whose twin was connected to one of the hooks at the end of the nerve. A change-over switch allowed the other hook to be connected instead. Both readings were taken for each setting of the nerve, and the percentage calculated. The current applied was a square wave of the same duration as that used in the experiments upon excitability, but of strength very much below threshold. An accurately measured fraction of the applied current was added to the cathode circuit of one of the cathode followers so that there was null output in the amplifier. This proved a very accurate and convenient method of measuring the interstitial potential, for the setting of this fraction is independent both of the current strength applied to the nerve (as we verified provided that it was not strong enough to produce an action potential) and to the linearity of the amplifier. And since the null condition is established at the amplifier's input, a great sensitivity may be used to record the balance without any danger of overload.

After the interstitial potential curve had been measured and confirmed, the nerve was removed from the apparatus, and the internal wire withdrawn and its insulation retested. The nerve was then cleaned of jelly, and treated as has already been described in Part 1 of this paper.

Theory

In Part 1 we have seen that stripped nerve behaves very much in accordance with the simple cable theory, and the fibres excited in Fig. 1 could be regarded as cables with $\lambda = 2{\cdot}8$ mm. having an excitability at each point proportional to the membrane depolarization. Let us now assume that in the experiments of Part 2 (which were made upon this same nerve before stripping) we are dealing with the same fibres, behaving in the same way. Then, since the difference in excitability seen in Fig. 2 is not to be attributed to a change within the fibres, it must be due to a change outside. This means that the presence of the epineurium has modified the potential distribution applied to the fibres. Can we calculate what modification would be required to produce Fig. 2? Certainly this can be done, but it is not precisely what we require.

If the interstitial potential calculated is to be directly comparable with that actually measured by the internal wire, we need to have the stimulus the same for both cases. The direct potential measurements were made using electrodes placed 1 mm. apart, consequently we need to analyse the spread of excitability around electrodes so placed. Now this is easily found from Fig. 2, for both theory (Rashbass & Rushton, 1949*b*) and experiment (Rashbass, 1949) have shown that the required excitability (the slot excitability curve) is given exactly by the differential of curve T, Fig. 2. This differential is plotted in Fig. 5, curve B, whose ordinates therefore show the excitability of the nerve at various distances on the cathodal side of an electrode pair placed at $x = \pm 0{\cdot}5$ mm. Now since excitability is proportional to membrane depolarization, the ordinates of curve B give the potential difference across the membrane at each point on a cable of $\lambda = 2{\cdot}8$ mm. There is only one external potential distribution which will give this membrane potential distribution, and we proceed to find it.

Let V_1 = interstitial potential at x, which we require to evaluate.

V_2 = potential at x in the axis cylinder of the fibres which are excited.

$1-\psi(x)$ = tripolar excitability curve T, Fig. 2 scaled to a maximum of unity.

r = resistance of axis cylinder per mm.

$\frac{1}{R}$ = conductivity of axon sheath per mm.

$\frac{R}{r} = \lambda^2 = 7{\cdot}8$ mm.^{2}.

$-a$ = arbitrary constant.

Now we have seen that the ordinates of curve B, Fig. 5, are proportional both to

$$\frac{d}{dx}[1-\psi(x)],$$

and to

$$\frac{V_2-V_1}{R} = \frac{1}{r}\frac{d^2V_2}{dx^2},$$

therefore

$$a\frac{d\psi}{dx} = \frac{V_2-V_1}{\lambda^2} = \frac{d^2V_2}{dx^2}; \tag{1}$$

therefore

$$V_2 = a\int_0^x \psi\, dx,$$

if we define as zero the potential at $x=0$. But from (1)

$$\begin{aligned} V_1 &= V_2 - a\lambda^2\frac{d\psi}{dx} \\ &= a\left[\int_0^x \psi\, dx - \lambda^2\frac{d\psi}{dx}\right]. \end{aligned} \tag{2}$$

Now $\psi(x)$ is plotted in curve A, Fig. 5. It is simply T, Fig. 2, upside down, and still scaled to a maximum of unity. Since ψ and λ^2 are exactly known, the value of V_1/a may be determined absolutely from equation (2) for all values of x.

The differentiation and integration of $\psi(x)$ was performed arithmetically as follows. Values of $\psi(x)$ measured up on curve A, Fig. 5, were tabulated one below the other for $x = 0{\cdot}5, 1{\cdot}5, 2{\cdot}5\ldots$ mm. in this order. Suppose that at $x=(n-\frac{1}{2})$ the ordinate value in the table is N, then we obtain $\left(-\frac{d\psi}{dx}\right)$ for $x=n$ by subtracting from N the value which lies immediately below it in the table. Also $\int_0^x \psi\, dx$ will be given by adding to N all the figures in the table which stand above it.

The curves B and C, Fig. 5, show the values of $\left(-\frac{d\psi}{dx}\right)$ and $\int_0^x \psi\, dx$ respectively calculated from the experimental curve A by this method. The unit of length is taken as the cm. in order that the representation may be at a convenient scale. Since $\lambda^2 = 0{\cdot}078$ cm.$^2 = \frac{1}{13}$, we must add to C, $\frac{1}{13}$ of B in order to obtain the required interstitial potential V_1/a.

Now it will be recalled that when the interstitial potential was directly measured, it was expressed as a percentage of the difference between the two ends of the nerve. Thus if V_1/a is to be plotted upon the same scale, the arbitrary constant a must be chosen so that the potential difference between the ends of the nerve amounts to 100.

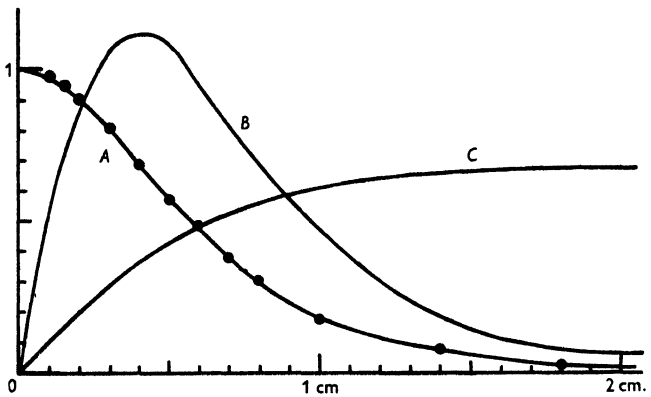

Fig. 5. Curve A is a replot upside down of T, Fig. 2; B is the negative differential of A; C is its integral. Unit of length = 1 cm. Curve A plots the distribution of excitability about an isolated cathode at O; B about an electrode pair at $\pm 0{\cdot}5$ mm.

Results

Since conditions are symmetrical, it is only necessary to represent the potentials in the various layers to the right of $x=0$, for those to the left will be equal and of opposite sign if we define zero potential as that at $x=0$, the mid-point between the electrodes. The curves of Fig. 6 are plotted in this way. They all pass through O and are scaled to have a value of 50 for large values of x. The electrodes are situated at $x = \pm 0{\cdot}5$ mm.

Curve V_0 shows the potential of the jelly outside the epineurium, and is a replot of Fig. 1 (Rashbass, 1949). We do not require it for our analysis, and it is included merely to complete the picture of the potential distribution in the nerve layers. Curve V_2 is the calculated potential in the axis cylinder of those fibres which are excited. It is a replot of curve C, Fig. 5. Curve V_1 shows the interstitial potential calculated as described above, e.g. by adding to C Fig. 5, $\frac{1}{13}$ of curve B.

It must be emphasized that the predicted interstitial potential V_1 has been calculated without any reference at all to the internal electrode. If our ideas were wrong, the actual interstitial potential might be any reasonable curve passing through O and running asymptotic to the 50 horizontal. If the sheath resistance were negligible, for instance, the curve would coincide with V_0. The actual potential measurements, however, are given by the black and white

circles of curve V_1, Fig. 6. The white circles are correctly placed, but the black ones belong to the left half of the symmetrical curve. They have been replotted in their corresponding places on the right side in order to show the degree of symmetry obtaining in this nerve, and to allow bothh alves to be compared with the computed curve V_1. It is seen that the curve falls only slightly below the points.

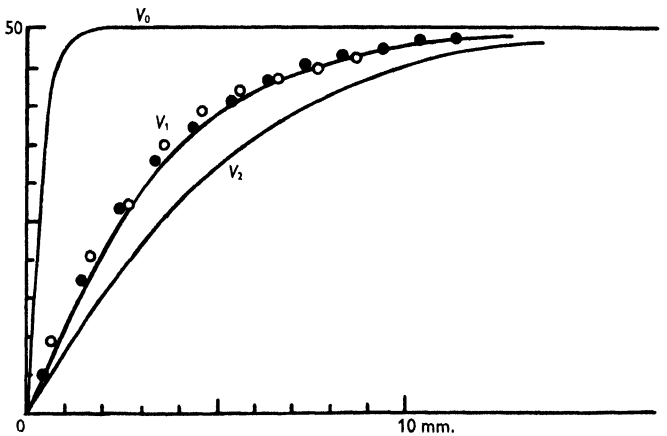

Fig. 6. The distribution of potential in the various layers of nerve to the right of an electrode pair situated at $\pm 0{\cdot}5$ mm. V_2 potential of core, a replot of C, Fig. 5. V_1, interstitial potential $= C + B/13$ (Fig. 5). V_0, surface potential. Black and white circles show the interstitial potential directly measured.

If it had not been possible to measure the interstitial potential directly, and we had been forced to rely entirely upon the calculations from excitability, we should have hesitated to accept a potential distribution where a far greater drop occurs across the epineurium than across the myelin. But the close confirmation by direct measurement leaves little doubt that the distribution of Fig. 6 is a fact. It also strengthens the theory which led to this agreement.

Is the resistance in the epineurium?

The epineurium holds the nerve fibres tightly packed in the trunk, so that it is reasonable to suppose that the fibres in the centre are screened by those nearer the outside. Stripping off the sheath certainly allows the fibres to separate, and so would abolish this screening. Is it certain, then, that what we have called the resistance of the epineurium is not in fact the resistance of the peripheral layer of nerve fibres, held compact by a quite permeable connective network? We incline to the view that epineurial resistance is the principal factor, for the following reasons:

(*a*) Fibre screening is only tenable if it is assumed that both excitability and potential measurements are made near the centre of the trunk. But it would

be surprising if the current excited easiest those fibres which were most screened from it. Moreover, potential measurements appeared to be the same no matter whether the bare recording area were deeply buried in the nerve, or so close beneath the epineurium that details of the scratches on the wire could be seen in the microscope.

(*b*) On several occasions a little slit was torn in the sheath just opposite the bare area of the wire when this was deeply situated in the trunk (one can always obtain the locality by measuring back from the visible tip). The potential of the wire which was initially at V_1, Fig. 6, jumped at once to V_0, and the potential curve measured in these conditions followed curve V_0 almost exactly. This effect of sheath puncture is naturally most marked at small extrapolar distances where the potential across the sheath, directly measured, is seen suddenly to fall to about 1% of its former value.

(*c*) In the early experiments when the sharp tip of the fine wire was left bare, it sometimes happened that the terminal portion got bent sideways so that the point stuck into the epineurium, raising it from the side of the nerve like a little tent on its pole. The recorded potential in such a case was about half-way between curves V_0 and V_1. Nothing but epineurial resistance can explain the potential drop in this case. And a low value is to be expected, because the stretching of the sheath and perhaps some penetration by the point may substantially diminish the resistance at the very place where it is measured.

Validity of the experiment

We have found this experiment very hard to perform owing to its delicate manipulations, its many accurate determinations, and the overall uniformity essential to the analysis. There is danger in such a case of prejudging the answer by being more ready to accept as 'good' those experiments which fit well, for one can generally find grounds for questioning the validity of results which turn out discrepant.

In order to avoid this, we considered all our principal sources of error and set a certain standard of performance. If that was not attained, this part of the experiment was at once rejected. If it was attained, this part was accepted, come what may. We have already mentioned the insulation test for the fine wire, and the criterion of nerve damage when the wire was inserted. All determinations were repeated in reverse order. The interstitial potential must be entirely reproduced within 2%, the thresholds within 5% and so on. The hardest test to satisfy was the uniformity of the epineurium as measured by the symmetry of the interstitial potential curve. Fig. 6 shows a nearly perfect symmetry, for the white dots and the transposed black dots practically fall upon the same curve. We never saw another curve as symmetrical as this, and usually they were quite bad. Now, the whole argument of this experiment is based upon the assumption of the uniformity of the sheath, and therefore

asymmetrical nerves could never be used either to prove the theory or to disprove it. All such experiments, therefore (and that is the majority) had to be rejected, in the sense that they could not be used to compare accurately the two methods of obtaining the interstitial potential. They could invariably be used to establish that the interstitial potential has a distribution like curve V_1 and quite unlike curve V_0.

In the course of this work our standards became more and more exacting as we discovered new sources of error and developed a better technique. It became rarer to succeed in passing all the tests, but the successful results approached closer to those we have described. It therefore appears that the curves of Fig. 6 represent the results to which all the experiments converge as their errors are progressively eliminated.

Only three experiments actually passed all the tests in their final form and the one here described was technically best, and also gave the closest correspondence between the calculated and observed interstitial potential. The other two were nearly as close, the deviation going hand-in-hand with sheath asymmetry. Thus though our valid experiments are very few, we believe that they are in fact valid experiments.

Discussion

The idea that the epineurium may have an appreciable resistance is an old one and based on three kinds of evidence—histological, pharmacological and electrical. The evidence from structure is simply that the nerve fibres are known to be surrounded by a denser outer sheath, the epineurium, and more numerous finer sheaths, the perineuria and endoneuria, and this raises the question whether such sheaths may affect the passage of electrolytes, but does little to answer it.

Diffusion. According to various authors, the epineurium is considered to be, or not to be a barrier to diffusion because of the rate of action of ions, narcotics, etc., applied to the nerve in various states of dissection. Tasaki (1939*b*), see also Kato (1936), found that narcotics applied directly to a node acted completely within 1 sec. or so, and we have witnessed the same thing in experiments by Huxley and Stampfli investigating the action of ions on frogs' single nerve fibres (unpublished as yet). A drop of sugar solution on the node abolished conduction, and a drop of Ringer restored it instantly and regularly. The action of these agents on unstripped nerve is some hundreds of times as slow, but it cannot be argued from this that the sheath is a barrier to diffusion and must have electrical resistance. There are two processes which contribute to the penetration of substances in solution, and since it is important to distinguish them, let us take a simple physical example.

A glass cylinder is half filled with $CuSO_4$, and above this is carefully run in isotonic Na_2SO_4 so there is a sharp line of demarcation between the two

solutions. Now diffusion is the penetration of solute to all parts of the solvent due to thermal agitation without any movement of the solvent. It would take some days to reach equilibrium in this example. Obviously the result could be achieved in a few seconds by vigorous shaking. In that case it is the agitation of the solvent which distributes the solute. Suppose that the solutions were set in jelly, the diffusion would be more or less unchanged, but, of course, shaking the jelly would not hasten mixing. Now the importance of this is that both *diffusion* and what we shall call *mixing* contribute to the penetration of substances, but only diffusion contributes to the electrical conductivity. So care must be exercised in arguing from penetration to electrical conductivity.

From these considerations we see that there is nothing inherently contradictory between Lorente de Nó's observation (1947*a*, p. 21) that 0·11 M solution KCl produces conduction block in 15 min. and his statement that the sheath does not act as a diffusion barrier (p. 23). For though the process takes perhaps a thousand times as long as it would by the direct application of the ions to a node, this might be explained simply by the absence of mixing. A stripped nerve set in jelly might show the same thing. It is harder to accept his conclusion that the action is so rapid that the sheath cannot possibly be a diffusion barrier. Indeed if this were so, we should expect that the outer layer of nerve fibres would be paralysed by a solution as strong as 0·1375 M within the first second, but no change can be detected for about a hundred times as long as this (p. 23).

A more direct approach is obtained by comparing results (*a*) with sheath intact, and (*b*) when removed either by dissection or by using the spinal roots. Rice & Davis (1928) found that chloral hydrate acted faster upon nerve in the region of cut branches, and paralysed the trunk more rapidly the more connective sheaths were cleaned away. Feng & Gerard (1930) found that methylene blue stained the sheath but would not penetrate, and that slitting the sheath permitted entry. They found that sheath-slitting accelerated conduction block by KCl, NaCN, $CaCl_2$, or glucose some ten times. For instance, KCl with intact sheath took 15 min.; without sheath less than 2 min. This striking result, however, is no evidence that the sheath is a *diffusion* barrier. It is undoubtedly a barrier to mixing, and the experiment does not prove that it is more.

The effect of mixing could easily be avoided by comparing stripped and unstripped nerves as in Feng & Gerard's experiments if both the nerves were set in jelly (rather thinner than in our experiments). Another way is to compare the penetration in the trunk and in the spinal roots, since the latter have hardly any epineurium but are probably held together compactly enough to stop much mixing. Bishop (1932, p. 182) in a footnote states that the same concentration of KCl acts much more quickly upon the roots than the trunk, and attributes this to the slow penetration through the frog's epineurium. Lundberg (1948) finds the same relation in the cat, where the penetration in

the roots was about 1000 times as fast as in the trunk. Since mammalian nerves have a thicker epineurium than frogs', and far more perineurial sheaths, Lundberg's results are not directly comparable with those from the frog, but they certainly favour the view that connective tissue is a barrier to diffusion.

Most of the evidence as to diffusion of reagents through the epineurium has been inconclusive because it has arisen as a complication in experiments directed to quite a different end. But it is hard to avoid the conclusion that the sheath is to some extent a diffusion barrier. Obviously a convincing experiment must secure penetration without mixing, must test the penetration by nerves of similar susceptibility, and must secure conditions of diffusion which are sensitive to sheath resistance—probably with a high concentration of reagent and a short penetration time. It is doubtful whether such a method of obtaining the epineurial resistance would be easier or more reliable than direct electrical investigation.

Current distribution. There can be very few of those working upon the relation of electricity to nerve activity who have not wondered how far the connective tissue might not contribute to the relations studied. In the literature, doubts usually come to expression when observations diverge from theory, but little is generally done to substantiate the question. Bishop *et al.* (1926), on the other hand, in their analysis of factors distorting action potential records, found the epineurium to be highly polarizable, as shown by comparing the potential distribution in the normal trunk either with the stripped trunk or the spinal roots. They express so clearly the implications of this, that it is surprising that physiologists have continued to neglect it. Bishop (1928*a*, *b*) developed the matter further, Cole & Curtis (1936) found that the transverse impedance of nerve was largely removed by careful stripping the sheath, and Tasaki (1939*a*) observed that the chronaxie of a single nerve fibre diminishes when the epineurium is removed, and then shortens still further when the single fibre is quite isolated.

These definite indications of epineurial influence are not seriously challenged by the great mass of work where a rather good correspondence has been observed with the predictions of the simple cable theory. The authors are usually content with establishing a formal relation, and make no pronouncement about the properties of the sheath. It is otherwise, however, with Lorente de Nó, who has recently expressed himself very strongly upon the matter.

'This fact (α), together with the other fact (β) that no electrotonic potentials can be produced after nerves have lost their core-conductor properties, constitutes conclusive evidence to warrant the statement that the existence of the connective tissue sheath may be ignored in the analysis of potentials recorded from the surface of nerve' (1947*a*, p. 13).

This statement leaves us no alternative but to attempt some discussion of the evidence, for not only is this quite contrary to our conclusions, but also our results, if accepted, would cast some doubt, we fear, upon many of Lorente de Nó's conclusions. It is certain that in the few pages that follow we shall do scant justice to his two volumes of interwoven argument. We can but state that we have tried to focus upon the impact point of our conclusions with his, and to appreciate as well as we can the line of his argument, in which we have been much assisted by a helpful and explanatory letter.

The fact which we have labelled (β) in the statement above constitutes by itself conclusive evidence if it is certain that the agents which abolish the core-conductor properties of axons are without effect upon the epineurium. If we do not know what the agents do to the epineurium, it is no evidence at all. Examples of loss of electrotonus are given (Lorente de Nó, 1949*a*, p. 405; *b*, pp. 174, 184) due to the action of K^+, iodoacetamide, and Cu^{++}. There is no physical evidence as to the action on the epineurium. The belief that its resistance did not fall to zero rests upon subsequent histological examination (osmic acid) where the membrane was seen to be normal. We are not histologists and no doubt underestimate what the microscope can reveal to one highly skilled in this science. But if the foregoing experiment had been done with a fine wire beneath the epineurium, and if the directly measured sheath potential had been seen to fall to zero, we should certainly accept this conclusion rather than the one derived from histology. Now the results of the suggested experiment may be reached without in fact performing it. For initially there is certainly a polarization potential across the epineurium, as we have invariably found, and at the end there is no polarization potential anywhere, as Lorente de Nó has found. It therefore seems plain that the epineurium suffers a resistance change without modification of histological structure.

The fact which we have labelled (α) is as follows. When a square current pulse is passed down a long uniform stretch of nerve, the potential recorded from two surface points in the middle region is at every instant proportional to the current (1947*b*, p. 13, fig. 11). From this it is reasonable to conclude that all the longitudinal conductors are ohmic (non-polarizable). It gives no information whatever about the sheaths through which the current passes radially because there *is* no current passing radially within range of the recording electrodes. We were therefore puzzled to know how this experiment could constitute Lorente de Nó's conclusive evidence that epineurial resistance is negligible. He has kindly indicated to us his line of argument, which again involves the histologist's viewpoint. It depends upon appreciating that the fine connective network—the fibrillenscheide of Key and Retzius—which surrounds the myelinated fibres throughout the trunk, has its fibres continuous with the epineurium. It is argued, therefore, that the absence of any polarization by the longitudinal current shows this network to be of non-polarizable

material, and hence that the epineurium, which is of the same material, is also non-polarizable.

This argument appears to be open to criticism of two kinds:

(i) *Electrical.* The observation of fact (α) proves that the longitudinal conductors have an ohmic resistance. If the fibrillenscheide is not part of the longitudinal conductor, it is impossible to argue anything about its non-polarizability. If it is part, its resistance must be ohmic. Suppose that it is not zero, then Lorente de Nó's argument leads at once to the result that the epineurium also has resistance. Thus the argument can hardly constitute conclusive evidence for the opposite opinion.

(ii) *Histological.* Before we can argue from the absence of polarization by longitudinal currents to the non-polarizability of the fibrillenscheide, we must be sure that these sheaths are in a condition to be polarized by this current, namely that they run transversely across the trunk, and are without gaps through which the current could by-pass them. But the fibrillenscheiden do not have this structure at all. Key & Retzius (1876, pl. VII (man), pl. IX (frog)), Cajal (1928, fig. 11, p. 63), Laidlaw (1930, fig. 1), de Rényi (1932, figs. 14, 15, pp. 1398–9), Nageotte (1932, fig. 3, p. 199), all agree in describing an exceedingly fine network closely investing the neurilemma, and as incapable of being polarized by a longitudinal current as the epineurium itself. From the descriptions of these authorities it would appear that longitudinal currents exhibit no polarization because no nerve structure exists for such a current to polarize. It is impossible, therefore, to argue from non-polarizability by longitudinal currents to any property of the epineurium.

Since a longitudinal current cannot and does not polarize anything, we may inquire what is the nature of Lorente de Nó's longitudinal polarization. From his mathematical treatment of it, it must be a vector with the dimension of potential gradient and direction longitudinal. It is generated within the nerve core in some way by current flow, but the author states definitely that it is not the polarization of core structure by current flowing longitudinally (1947*b*, p. 12). But it is by no means clear how current flowing radially could produce polarization longitudinally, nor why this should be directed up the nerve rather than down, at any given point. 'Under conditions such as these it is indeed regrettable that the experimental observations do not throw light on the mechanism underlying the longitudinal polarization of the nerve fibres' (1947*a*, p. 100). 'Since the longitudinal electromotive forces that appear in the core of the nerve fibres are not accessible to direct measurement, the only possibility of establishing their existence consists in demonstrating that the predictions of "equation (2)" do not agree with the experimental results' (1947*a*, p. 246).

Now, 'equation (2)' is a relation derived from the simple cable theory which we have seen does not in general apply to unstripped nerves such as were used in Lorente de Nó's experiments. When our nerves were stripped they appeared

to satisfy pretty well the predictions of 'equation (2)', and if this happened with Lorente de Nó's nerve, removal of the sheath would also remove his 'longitudinal polarization'. We believe this likely on analytical grounds.

Consider his fig. 1 (1947*a*, p. 3), which plots the distribution of the demarcation potential along the nerve. The curve is seen to resemble our curve *A*, Fig. 5, which plots the distribution of excitability about the cathode—the single-pole excitability curve (Rashbass & Rushton, 1949*b*). In both cases an exponential is to be expected upon the simple cable theory, and in both cases we observe the same kind of deviation from expectation. The resemblance is more than coincidental, for the formal identity of the two curves may be established and that independent of any special assumption as to structure.

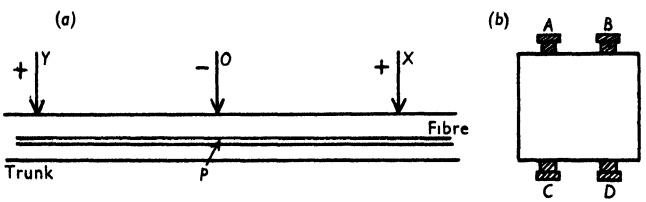

Fig. 7

Fig. 7 (*a*) shows a nerve trunk arranged for symmetrical tripolar stimulation, and within, one of the nerve fibres excited. This is all the structure that is assumed. We shall now show the relation between the spread of excitability and the spread of demarcation potential, by applying Rayleigh's Reciprocity theorem. This theorem may be stated simply as follows. The box shown in Fig. 7 (*b*) contains any kind of electrical network in which the Superposition theorem holds, and the wires from terminals *A*, *B*, *C*, *D* connect to four points anywhere in the network. A current i sent in through *AB* will generate a voltage V between *C* and *D*. Then the theorem states that if this same current i is sent through *CD*, it will generate the same voltage V between *A* and *B*.

Now curve *T*, Fig. 2, shows the symmetrical tripolar excitability for various interelectrode distances x. The ordinate of this curve $1-\psi(x)$ represents therefore the depolarization produced across the axon membrane at *P*, Fig. 7, by a fixed current pulse applied through the anodes (*X* and *Y*) for various values of x. But, by the Rayleigh theorem, if a fixed current sent in through (*X* and *Y*) and out through *O* produces at *P* a potential difference $1-\psi(x)$ across the membrane, then a fixed demarcation current across the membrane at *P* will produce a potential difference of $1-\psi(x)$ between *O* and (*X* and *Y*). Or, measuring the demarcation potential between *X* and a distant point to the right (as in Lorente de Nó's fig. 1), we obtain the curve $\psi(x)$ which is plotted in *A*, Fig. 5. The similarity of these two curves is therefore by no means fortuitous.

Now it is precisely to account for the fact that the demarcation curve is not exponential, that Lorente de Nó first introduces the postulate of longitudinal polarization. We have just seen that another interpretation is possible, for the epineurium whose resistance exactly explains the shape of curve *A*, Fig. 5, also leads us to expect the same shape for the demarcation potential curve. But we may go further. Since the formal equivalence of excitability spread and demarcation spread does not depend upon any assumption as to structure, the correspondence occurs equally in stripped nerve. Now we have seen (Fig. 1) that by stripping, we reduce the excitability spread to the theoretical exponential, hence the demarcation spread will also in this case be a simple exponential. If this reasoning is valid, therefore 'longitudinal polarization' will be abolished by stripping off the epineurium.

It will be observed that the foregoing formal argument neglects the following: (i) that the type of fibre excited at one quarter maximal response may not be the same as that responsible for the demarcation potential; (ii) that one of the nerves compared was in jelly and the other in air; (iii) that the demarcation potential was probably produced by a method which also damaged the epineurium; (iv) that the Superposition theorem does not always hold exactly for nerve.

It is plain that many of the phenomena which Lorente de Nó attributes to longitudinal polarization could not be explained simply by taking into account the resistance of the sheath. But since some of the results seem to be completely explicable in this way, and since longitudinal polarization itself is not a very precise concept, we feel doubtful whether it is the most fruitful way of explaining the others.

CONCLUSIONS

This paper, together with the four which preceed it, form a connected study of the distribution of excitability in frog's nerve, and we have now cleared up most of the points left outstanding in our former work. Starting by proving that when anode and cathode lie close together, excitation does not arise at the manifest cathode but at some point 3 mm. away (Rushton, 1949), we investigated the distribution of excitability in this case (the slot excitability curve), and found that it did not fall away from the cathode along an exponential curve, but had the form shown in *B*, Fig. 5, with its maximum 3 mm. extrapolar (1949*a*). We now see that the shape of this curve is exactly that required by the simply cable theory as applied to the axons themselves, and Fig. 6 shows clearly how it comes about that depolarization ($= V_1 - V_2$) is maximal at 3 mm. extrapolar. Stimulation by a single pole (1949*b*) gave the distribution of excitability shown here in *A*, Fig. 5, which is exactly the negative integral of *B* (Rashbass, 1949). If this curve has been determined, we may predict the excitability of each point on a uniform nerve for any given distribution whatever of the stimulus, by application of the Superposition theorem (1949*b*).

If a stripped nerve has any given potential applied to the outside the potential of the core may be found most simply by the graphical method of subtangent analysis (Rushton, 1937). If the external potential curve is analysed to the right with subtangent λ and the curve so obtained then analysed to the left in the same way, the resulting curve gives the potential of the core at each point. Each analysis takes about a minute to do.

Now a bundle of uniform nerve fibres contained in a uniform epineurium may be treated in a similar fashion. In this case there are two constants λ_1 and λ_2, and the subtangent analysis must be performed in both directions with each subtangent. If this is done upon the external potential distribution V_0 of the present paper, by choosing λ_1 and λ_2 suitably the curve C, Fig. 5, may be obtained with an accuracy within the thickness of the ink line. It follows that we may obtain the core potential of the fibres concerned in Fig. 5 for any given external potential distribution whatever, by analysing this given curve using subtangents λ_1 and λ_2. The excitability curve is, of course, the second differential of the core potential.

The validity of the analysis does not depend upon the assumption that all the fibres in the nerve are uniform (which is certainly not true). It depends upon the fact that a suitable choice of λ_1 and λ_2 does exactly generate the single pole excitability curve A, Fig. 5, and hence it must also give all the results which could be obtained by applying to A the Superposition Theorem. It will therefore give the right answer, but one cannot safely argue back to the significance of λ_1 and λ_2.

The localization of excitation in uniform nerve follows at once from the foregoing, for threshold excitation will arise at the place where the excitability curve has its maximum. The maximum is always rather flattened, and the sharpest condition easily realized is when a cathode has an anode close on either side. The excitability curve in this case is the differential of curve B, Fig. 5, and even here the nerve is catelectrotonic for 3 mm. on either side of the cathode, however close the anodes may be. The excitation site will be displaced away from an isolated cathode when the anode approaches within 10 mm., the relation being shown in (Rashbass & Rushton, 1949*b*) Fig. 3, curve L. If the nerve is not uniform because of the nodes or other factors, the excitation site may be some distance from the exact maximum on the rounded excitability hump. Plotting according to the method of Fig. 3. (present paper) probably gives the most accurate information as to the precise excitation site.

Electrotonic potentials

It is plain from Fig. 6 that it is not safe to assume either that the potential difference between the outside (V_0) and the nerve core (V_2) is all occurring across the nerve membrane, or that all the current which flows down the core passes back outside the epineurium, for there is also interstitial current. It is true that this nerve, mounted in jelly, is not quite comparable with a nerve in air as used in measurements of electrotonus. However, though we have not thoroughly analysed the latter condition, Rushton has demonstrated to the Physiological Society (unprinted) two significant observations. (*a*) A fine wire was inserted beneath the epineurium, and the potential difference was recorded between this and a distant point of nerve when a square polarizing current of 2 msec. was applied in the usual way. If the bare area of the fine wire was exactly under the cathode (within 0·2 mm.) there was zero electrotonic

potential recorded, but a large action potential appeared if the polarizing current exceeded threshold. Electrotonic potentials of either sign could be obtained by displacing the fine wire one way or the other in relation to the polarizing electrode. The potential across the epineurium at the cathode was of course very large. It will suffice here to say that an investigation of the potential distribution on either side of the epineurium in those experiments is entirely consistent with the expectations of this paper, and may be calculated approximately from the double cable theory. (*b*) When the epineurium is stripped off, electrotonus is very small. Measured between the cathode site and a distant point when the stimulus was just sufficient to give a maximal action potential, the electrotonus was less than 5% of the spike height. This confirms the impedance measurements of Cole & Curtis (1936), where, after careful removal of the sheath, the transverse resistance was found to drop to one-fifth of its previous value.

It is clear that the presence of the epineurium is a formidable complication to measurements of electrotonus. Preliminary work to analyse this was discontinued when we found how hard it was to obtain a nerve with a uniform sheath. Such analysis is in any case rather academic, since investigations of electrotonus in future are bound to be conducted upon stripped nerve.

Nearly a quarter of a century ago Bishop *et al.* appreciated very clearly the essential content of this paper, and it is appropriate to end by quoting their conclusions (1926, p. 607).

'Further, our experiments tend to show that the sheath polarization is a large component of the "escape", and analogously, must produce distortion of the nerve's own potential as recorded. It, together with the high longitudinal resistance of nerve, tends to mask any change that may occur in the polarizability or permeability of the nerve with excitation. It also tends to make the effective contact of an electrode with the nerve, however narrow, broader than the actual electrode surface. Finally it must profoundly affect electrotonus, as measured, in the same manner as it affects escape....Thus most of the artifacts in the nerve records discussed above reduce to effects of the presence of, or alterations in the condition of, a structure which is not nerve at all. We cannot avoid the suspicion that much of the work on nerve resistance and permeability, electrotonus and polarization, liminal gradients, etc., needs reinterpretation in the light of the possible effects of a highly polarizable resistance being interposed between the nerve axon and the electrode.'

Summary

1. A method is described for removing the epineurium from the frog's sciatic (p. 110) which appears not to damage the majority of the nerve fibres (p. 117 (*a*)).

2. After stripping, the bipolar and tripolar strength-length curves (Fig. 1) are found both to fit closely the curve $1-e^{-x/2\cdot 8}$. Before stripping this nerve, the curves were as shown in Fig. 2.

3. The small discrepancies which remain after stripping may be due to residual connective tissue, or to the distribution of the nodes of Ranvier about the cathode.

4. Fig. 3 shows the results of asymmetric tripolar stimulation. The curves approximate to the triangle obtained by Tasaki when electrodes were applied to three consecutive nodes.

5. Anodal 'break' excitation and the anodal block of conduction are much increased by stripping so that these phenomena appear at current strengths low enough to complicate 'make' threshold measurements (see diagram, Fig. 4). We cannot explain this.

6. If the epineurium has a resistance, the potential of the interstitial fluid within will be quite different from that applied to the outside of the nerve. Part 2 describes an experiment to find out whether the difference between Figs. 1 and 2 is due entirely to this cause. It may be determined as follows:

(*a*) It is possible to calculate uniquely what the interstitial potential must be to account for the curves of Figs. 1 and 2. The result is curve V_1, Fig. 6.

(*b*) The interstitial potential may be measured directly by inserting a fine electric probe beneath the epineurium. The results are the black and white dots which lies close to curve V_1, Fig. 6.

7. The curves V_0, V_1, V_2, of Fig. 6 show respectively the potentials of the exterior, the interstitial fluid, and the core of the fibres excited in Figs. 1 and 2. There is generally a greater potential drop across the epineurium than the myelin.

8. Lorente de Nó's view that the epineurium does not affect current distribution is discussed. His arguments are criticized, and his 'longitudinal polarization' shown to be largely a manifestation of the polarization of the sheath which he had neglected.

9. The bearings of epineurial resistance upon the electrophysiology of nerve are summarized.

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SURFACE-ACTIVE SUBSTANCES AND THE LIBERATION OF ENZYMES FROM RABBIT POLYMORPHONUCLEAR LEUCOCYTES

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Much can be learned of the function of the white cell by studying its constituent enzymes. Our present knowledge of white-cell enzymes has been reviewed in papers by Barnes (1940) and Rebuck (1947). Such an obvious experimental approach to the understanding of the physiology of leucocytes has been neglected for so long because first, cell suspensions containing one cell type only are difficult to prepare and secondly, when such a cell suspension has been prepared, it is difficult to obtain from it an active enzyme solution, not contaminated with cells or cell fragments.

Cell-free preparations, suitable for studying the enzymes of the rabbit polymorphonuclear leucocyte, have been obtained by adding various surface-active substances to suspensions of the cells. The technique has proved useful in studying the phosphatases (Cram & Rossiter, 1948, 1949), esterases (Rossiter & Wong, 1949) and, more recently, the β-glucuronidases of rabbit polymorphonuclear leucocytes. A preliminary report of these experiments has already appeared (Rossiter, 1949).

METHODS

Cell preparations. Polymorphonuclear leucocytes were obtained from the peritoneal cavity of the rabbit by the method of de Haan (1918). A warm sterile solution of 0·9% NaCl (300–500 ml.) was run into the peritoneal cavity and drained off 3–4 hr. later into a beaker containing a few grains of heparin. Alternatively, the solution was left in the animal overnight and a further quantity was run in the following morning and withdrawn immediately. In each instance the resulting opalescent fluid contained 2000–16,000 leucocytes/cu.mm., at least 95% of which were polymorphonuclear.

Alkaline phosphatase estimation. Alkaline phosphatase was determined by the method of King & Armstrong (1934) in which the phenol, liberated by the hydrolysis of disodium phenyl phosphate, is estimated by the method of Folin & Ciocalteu (1927). Sufficient magnesium chloride was added to each tube to make the final magnesium concentration 0·001 M. Details of the test have been published by Cram & Rossiter (1949). The results were recorded as the amount of phenol (in mg.) liberated, under the standard conditions of the test, by 100 ml. enzyme in 1 hr.

Esterase estimation. Esterase was determined by the method of Rona & Lasnitzki (1924) in which the CO_2, displaced from a bicarbonate buffer by the hydrogen ions liberated during the hydrolysis of tributyrin, is measured in a standard Warburg manometer. Details of the test have been published by Rossiter & Wong (1949). The results were expressed as the volume of CO_2 (in ml.) liberated by 100 ml. enzyme solution in 1 hr.

RESULTS

Experiments with alkaline phosphatase

Saponin. Table 1 shows that saponin, added to make the final concentration 0·1 %, increased the alkaline phosphatase activity of suspensions of rabbit polymorphonuclear leucocytes. In the absence of saponin the substrate could not, because of the limited permeability of the cell membrane to disodium phenyl phosphate, reach the active centres of the enzyme within the cell. After saponin had been added, the substrate was available to the enzyme, and maximum activity resulted.

TABLE 1. Effect of saponin on the alkaline phosphatase of suspensions of washed rabbit polymorphonuclear leucocytes. Substrate, disodium phenyl phosphate 0·004 M; magnesium concentration, 0·001 M; saponin concentration, 0·1 %; incubation time, 1 hr.; temperature, 37°

Phosphatase activity (mg. phenol/100 ml./hr.)		Relative activity in presence of saponin
No saponin	Saponin 0·1 %	(%)
3·9	9·0	231
5·6	12·0	214
18·0	56·0	311
18·3	36·0	197
24·6	36·0	147
	Mean (±S.D.) =	220·0 (±53·5)

That the enzyme was liberated from the cell is shown by the experiments reported in Table 2. One sample of cells was suspended in 0·9 % NaCl and a similar sample in 1 % saponin in 0·9 % NaCl. After the alkaline phosphatase activity had been determined in each, the two suspensions were centrifuged. The resulting cell-containing residues were made up to their original volume with 0·9 % NaCl. The alkaline phosphatase activity of each of the two residues and each of the two cell-free supernatants was then determined. For the cell originally suspended in 0·9 % NaCl, the greater part of the enzyme activity remained in the cell-containing residue and little was in the supernatant, but for the cells originally suspended in 1 % saponin in 0·9 % NaCl, the greater part of the activity was in the cell-free supernatant, very little remaining in the cells. Moreover, when 1 % saponin was added to the residue of the cells that previously had been suspended in 0·9 % NaCl only, the full activity of the saponin suspension or saponin supernatant was again restored.

The quantitative aspects of saponin activation are demonstrated in Fig. 1. To each of a series of centrifuge tubes containing the spun cells from 1 ml. of suspension was added 1, 5 or 10 ml. of a solution of saponin in 0·9 % NaCl. The

concentration of the saponin solutions varied from 0·05 to 1 %. After the tubes had stood for 30 min. at room temperature, one-fifth of the total volume (i.e. 0·2 ml. from the tubes to which 1 ml. had been added, 1 ml. from the tubes to

TABLE 2. Effect of 1 % saponin on the distribution of alkaline phosphatase between cells and supernatant of suspensions of washed rabbit polymorphonuclear leucocytes. Substrate disodium phenyl phosphate 0·004 M; magnesium concentration, 0·001 M; incubation time, 1 hr.; temperature 37°

	Phosphatase activity (mg. phenol/100 ml./hr.)					
	Preparation 1			Preparation 2		
Cells suspended in	Original suspension	Supernatant	Cells	Original suspension	Supernatant	Cells
0·9 % NaCl	4·2	0·5	3·4	1·4	0·03	2·0
1 % saponin in 0·9 % NaCl	25·7	23·6	6·4	13·6	11·8	2·6
Isotonic saline. Cells centrifuged off and resuspended in 1 % saponin in 0·9 % NaCl	—	—	25·2	—	—	10·6

which 5 ml. had been added, etc.) was removed from each tube for a determination of alkaline phosphatase. For concentrations of saponin above 0·5 %, maximal activity was observed no matter what volume of saponin solution had been added; the cells suspended in 1 ml. had a similar activity to those suspended in 5 or 10 ml. For concentrations of saponin less than 0·5 %, the enzyme activity was greatest in the cells that had been suspended in 10 ml. saponin solution and least in those that had been suspended in 1 ml., i.e. the enzyme activity was greatest when the ratio of the absolute amount of saponin to the white-cell concentration was greatest.

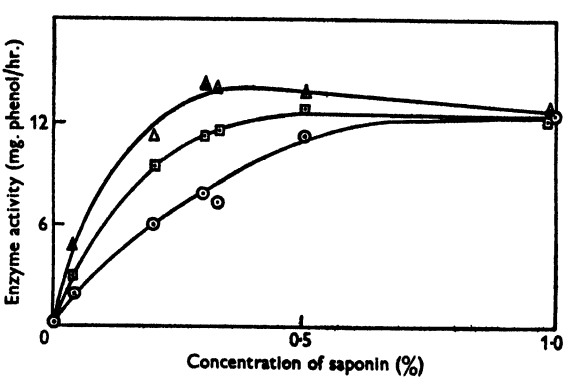

Fig. 1. Liberation of alkaline phosphatase from rabbit polymorphonuclear leucocytes by saponin. Cells from 1 ml. of suspension suspended in 1 ml. (⊙), 5 ml. (⊡), or 10 ml. (△) saponin solution. Each tube centrifuged and enzyme activity determined in one-fifth of total volume of supernatant.

In Fig. 2 the data of Fig. 1 are plotted with mg. saponin per tube, regardless of total volume, as abscissa and enzyme activity as ordinate. The activity of

the enzyme was greatest when the absolute amount of saponin in each tube was greatest and was independent of the concentration of saponin. This would suggest that the amount of saponin necessary to produce full enzyme activity is related stoichiometrically to the concentration of white cells. Fig. 2 shows that 20 mg. saponin was sufficient to produce maximum activity in 1 ml. cell suspension. In the experiments reported in Table 1 the proportion of saponin to cell suspension was 0·5 ml. 1 % saponin to 0·2 ml. cell suspension, or 25 mg. saponin to 1 ml. suspension. This is well in excess of the amount necessary to activate the enzyme fully.

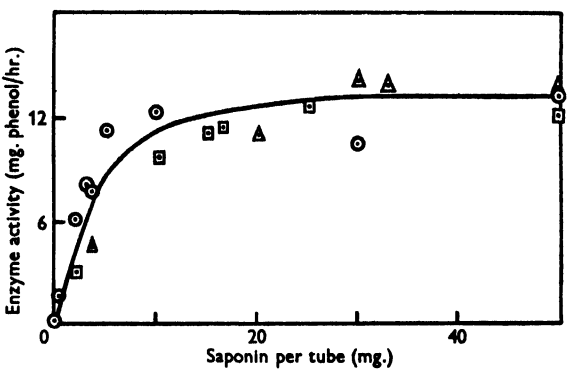

Fig. 2. Same data as in Fig. 1, but plotted with mg. saponin per tube as abscissa rather than the concentration of the saponin solution. For further explanation see legend to Fig. 1 and text.

Alkyl sulphate. Alkyl sulphate is a highly active anionic detergent obtained commercially. Like saponin it increased the alkaline phosphatase activity of suspensions of rabbit polymorphonuclear leucocytes (Table 3). For the concentration of cells usually obtained from the rabbit peritoneal cavity 0·06 % alkyl sulphate gave a maximal effect (Fig. 3). Unlike saponin, higher concentrations of alkyl sulphate greatly inhibited the enzyme, possibly because alkyl sulphate has a strong protein denaturing action (Putnam, 1948). In the experiments reported in Table 3, 0·06 % alkyl sulphate was used, but the peak of the alkyl sulphate-activity curve is extremely steep; it is possible that, in all instances, alkyl sulphate was not present in optimal concentrations. This is suggested by the observation that the mean relative activity in the presence of alkyl sulphate (Table 3) was somewhat less than that for 0·1 % saponin (Table 1). For this reason, when the alkaline phosphatase of white cells was determined quantitatively, saponin was always used.

Experiments similar to those reported in Table 2 showed that alkyl sulphate, like saponin, liberated phosphatase from the cells into the surrounding fluid.

TABLE 3. Effect of alkyl sulphate on the alkaline phosphatase of suspensions of washed rabbit polymorphonuclear leucocytes. Magnesium concentration, 0·001 M; substrate, disodium phenyl phosphate 0·004 M; incubation time, 1 hr.; temperature, 37°

Phosphatase activity (mg. phenol/100 ml./hr.)		Relative activity in presence of alkyl sulphate (%)
No alkyl sulphate	Alkyl sulphate 0·06%	
5·8	15·2	262
8·3	14·3	173
9·0	14·5	161
9·8	14·7	150
	Mean (±S.D.) =	186·5 (±44·3)

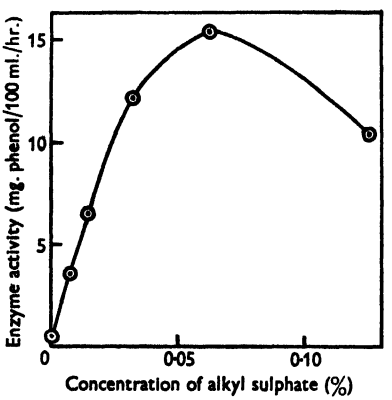

Fig. 3. Liberation of alkaline phosphatase from rabbit polymorphonuclear leucocytes by alkyl sulphate.

TABLE 4. Effect of sodium taurocholate on the alkaline phosphatase of suspensions of washed rabbit polymorphonuclear leucocytes. Substrate, disodium phenyl phosphate 0·004 M; magnesium concentration, 0·001 M; incubation time, 1 hr.; temperature 37°

Concentration of sodium taurocholate (M)	Phosphatase activity (mg. phenol/100 ml./hr.)		Relative activity in presence of bile salt (%)
	No bile salt	Bile salt	
0·00125	3·2	8·0	248
0·00125	4·6	9·9	218
0·00125	5·8	14·9	257
0·00125	9·1	24·9	274
		Mean (±S.D.) =	249·2 (±20·3)
0·00625	5·7	10·6	186
0·00625	10·4	21·2	203
0·00625	11·1	21·2	191
0·00625	13·3	22·2	167
0·00625	44·1	61·2	139
		Mean (±S.D.) =	177·2 (±22·3)

Bile salts. Table 4 shows that bile salts also increased the alkaline phosphatase activity of suspensions of rabbit polymorphonuclear leucocytes. In Table 4 the effect of sodium taurocholate only is given, but similar results were obtained

with sodium glycocholate and sodium desoxycholate. The relative activity in the presence of 0·00125 M-sodium taurocholate was greater than that observed when the concentration was 0·00625 M. This was to be expected, for previously 0·00625 M-sodium taurocholate was found to inhibit the alkaline phosphatase of cell-free preparations from rabbit polymorphonuclear leucocytes to the extent of about 35% (Cram & Rossiter, 1949).

Experiments similar to those reported in Table 2 showed that bile salts also liberated the enzyme from the cells.

Experiments with esterase

Saponin. Fig. 4 shows the esterase activity of a suspension of rabbit polymorphonuclear leucocytes. A similar suspension was separated by centrifuging into a cell-free supernatant and a cell-containing residue, which was made up

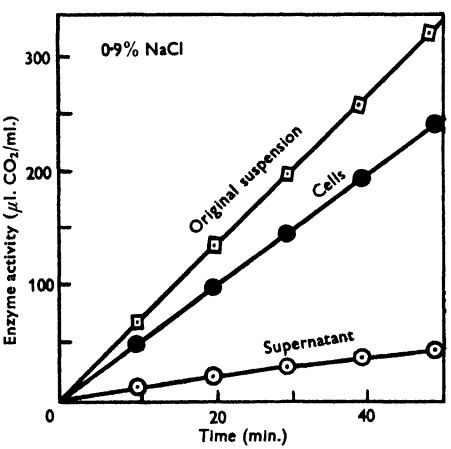

Fig. 4.

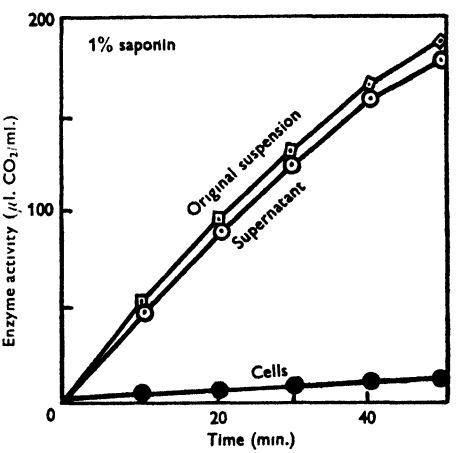

Fig. 5.

Fig. 4. Effect of centrifuging on the esterase activity of a suspension of rabbit polymorphonuclear leucocytes in 0·9% NaCl.

Fig. 5. Effect of centrifuging on the esterase activity of a suspension of rabbit polymorphonuclear leucocytes in 1% saponin in 0·9% NaCl.

to the original volume with 0·9% NaCl. Most of the activity remained in the cells, very little being found in the supernatant. For cells suspended in 1% saponin in 0·9% NaCl the reverse was true (Fig. 5). Most of the activity was found in the supernatant, very little remaining in the cells. The esterase, like the phosphatase, was liberated from the cells into the surrounding fluid.

The esterase activity of the saponin supernatant was similar to that of the original suspension. This was unlike the phosphatase, where the enzyme activity of a cell-free preparation was much greater than that of the original cell suspension. Tributyrin, unlike disodium phenyl phosphate, the substrate for the

phosphatase, was presumably not limited by the relative impermeability of the cell membrane, and could reach the active enzyme centres within the cell, even in a suspension of intact cells.

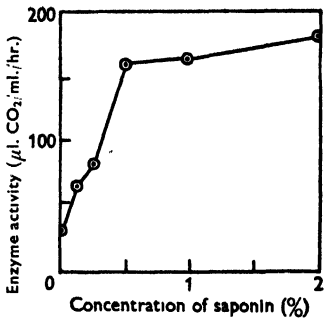

Fig. 6. Liberation of esterase from rabbit polymorphonuclear leucocytes by saponin.

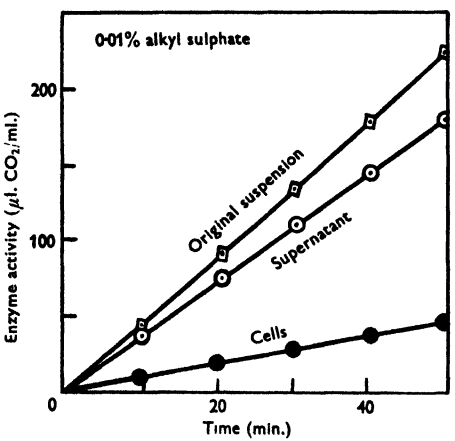

Fig. 7.

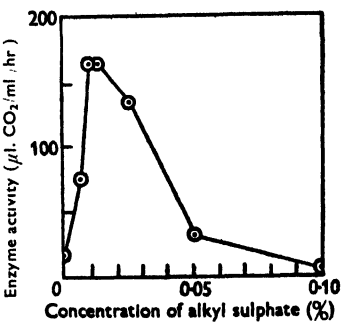

Fig. 8.

Fig. 7. Effect of centrifuging on the esterase activity of a suspension of rabbit polymorphonuclear leucocytes in 0·01% alkyl sulphate in 0·9% NaCl.

Fig. 8. Liberation of esterase from rabbit polymorphonuclear leucocytes by alkyl sulphate.

In the foregoing experiments the concentration of saponin was 1%. Fig. 6 shows the activity of a cell-free saponin supernatant plotted against the concentration of the saponin solution in which the cells originally had been suspended. For the cell concentration used, 0·5–2% saponin gave maximal activity.

Alkyl sulphate. Alkyl sulphate behaved similarly to saponin (Fig. 7). Fig. 8 shows that the concentration of alkyl sulphate was critical. For the esterase, as for the phosphatase, low cencentrations of alkyl sulphate failed to produce maximal liberation of the enzyme from the cell, while high concentrations were inhibitory.

Bile salts. Other experiments, similar to those of Figs. 5 and 7, showed that bile salts also liberated the esterase from the cells.

DISCUSSION

That surface-active substances, such as saponin or bile salts, lyse red blood cells has long been known. Recently Ponder (1946) studied the effect of a series of anionic detergents on red blood cells. The action of surface-active substances on white cells, however, has received little study. Ponder & Macleod (1936) showed that a number of surface-active substances depressed the respiration of rabbit polymorphonuclear leucocytes, an observation confirmed in this laboratory. They assumed the decrease in respiration to be an index of cytolysis. If the respiratory enzymes were, like phosphatase or esterase, liberated from the cells, it is likely that they would no longer be active. On the other hand, the respiratory enzymes might have been inhibited by the surface-active substance itself, for Hockenhull (1948) showed that a number of detergents, both anionic and cationic, inhibited the succinic oxidase system. My experience with phosphatase and esterase, and latterly with β-glucuronidase, tends to confirm the interpretation put on their results by Ponder & Macleod (1936).

There can be little doubt that substances such as saponin, bile salts, or alkyl sulphate liberate certain enzymes from the cell. It should be pointed out, however, that this process is not an all-or-nothing phenomenon as is the lysis of red cells. After the cells have been exposed to the surface-active substance they still possess a recognizable structure. The cell membrane remains, but the cytoplasm appears clear and gives the impression that much of the cytoplasmic protein may have left the cell. The cell nucleus is greatly enlarged and, at times, is fragmented. Cell counts show that the total number of cells do not diminish.

Surface-active substances would appear to have some use in extracting enzymes from cells. The technique is obviously limited to such enzymes as are not inhibited by the surface-active substance. Bile salts have been shown to inhibit the alkaline phosphatase of rabbit polymorphonuclear leucocytes and excess alkyl sulphate inhibits both the alkaline phosphatase and the esterase. Such an inhibitory action is not surprising, since many synthetic detergents combine with proteins and may even denature them (Putnam, 1948). Nevertheless, surface-active substances *per se* do not necessarily inhibit enzymes. Marron & Moreland (1939) found no correlation between the surface-tension lowering properties of a number of surface-active substances, including one synthetic detergent, and their ability to inhibit such enzymes as phosphatase, urease, or tyrosinase.

Saponin appears to be less inhibitory than many of the synthetic detergents.

SUMMARY

1. Surface-active substances, such as saponin, alkyl sulphate, or bile salts, increase the alkaline phosphatase activity of suspensions of rabbit polymorphonuclear leucocytes.

2. Saponin acts by liberating the enzyme from the cell. The amount of saponin necessary is related stoichiometrically to the number of cells in the suspension.

3. The same surface-active substances also liberates esterase from rabbit polymorphonuclear leucocytes.

4. High concentrations of alkyl sulphate or bile salt, but not of saponin, inhibit the enzymes studied.

5. The use of this technique for measuring quantitatively the concentration of enzymes in white blood cells and for obtaining cell-free preparations for the study of white-cell enzymes is discussed.

Much of this work was carried out in collaboration either with Dr D. M. Cram or Miss Esther Wong. It was aided by a grant from the National Research Council of Canada.

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THE NICOTINIC ACTION OF SUBSTANCES SUPPOSED TO BE PURELY SMOOTH-MUSCLE STIMULATING. (A) EFFECTS OF α-β-ETHYLAL-γ-TRIMETHYL-AMMONIUM-PROPANEDIOL (2268 F) UPON SKELETAL MUSCLE AND GANGLION CELLS

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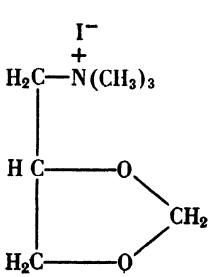
The quaternary iodide of α-β-ethylal-γ-trimethylammonium-propanediol (2268F) was synthesized by Fourneau, Bovet, Bovet & Montezin (1944), with the idea that it might be identical with natural muscarine. They reached the conclusion that 2268F differs from natural muscarine in certain respects, although it probably bears a structural resemblance to it, and has similar pharmacological properties. These consist, in the main, of a series of intense parasympathomimetic actions on the heart and blood vessels, on salivary secretion, and on various smooth muscles. When compared with acetylcholine it is about 10–100 times more powerful weight for weight. This is attributed by Fourneau *et al.* to the greater stability of 2268F which, they state, is not hydrolysed by cholinesterase. These actions of 2268F, and particularly its depressor effect, are antagonized by atropine, but the same authors found no evidence, in atropinized dogs, of a second, pressor, action, like that of acetylcholine, the so-called 'nicotinic' action—although the closest homologue to



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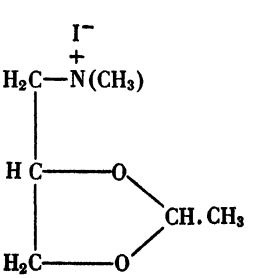

2268 F

2268F, namely 2249F (formal-γ-trimethylammonium-propanediol), which only differs from it by a-CH_2 group, is said to possess a distinct nicotinic activity.

Pannier & Verbeke (1947) have found that 2268F stimulates the respiratory centre directly in small doses (0·1 μg./kg.), and depresses it in larger amounts. In addition, the same dose (0·1 μg./kg.) indirectly affects the respiratory centre through reflexes originating in the carotid sinus and aortic regions, probably as the result of stimulation of chemoreceptors. Likewise, this substance has both reflex and direct actions on the vasomotor centre in the brain. Thus it is quite clear from these authors' observations that 2268F can exert a powerful influence upon certain types of nerve cell. This, however, together possibly with some of its action on the frog's rectus abdominis (Fourneau, *et al.* 1944) is an effect which cannot conveniently be classified as 'muscarinic' in the accepted sense.

This drug has also been used by Emmelin & Feldberg (1947) in crucial 'control' experiments on the gut, on the assumption that it is a purely muscle-stimulating substance, entirely free from any additional effect upon the nervous structures within the gut. Because of the importance of this assumption, and of the suspicion that 2268F may in fact also stimulate nerve cells, it seemed desirable to examine the action of this compound upon an isolated preparation of nerve cells, such as is found in the superior cervical ganglion. The salient fact which emerges from this work is that, besides possessing other nicotinic actions on various skeletal muscles, 2268F, when injected into the superior cervical ganglion in very small amounts during a perfusion, is capable of stimulating the ganglion cells. Since this action is antagonized by small doses of atropine, it is preferable to refer to it, not as 'nicotinic', despite the general resemblance, but as ganglion cell stimulating.

METHODS

Frog rectus abdominis and dorsal muscle of the leech. The preparations were suspended in a 3 c.c. bath, in Locke solution diluted with distilled water in the ratio of 1 : 1·4. The Locke solution was not eserinized, except in one experiment. It was found that, with frequent changes of bath fluid, aeration could be dispensed with. Drugs were administered at intervals of between 5 and 10 min.

Avian iris. Domestic laying hens were used. The reaction to light was tested in both eyes. Two drops of 4% cocaine hydrochloride were instilled into one conjunctival sac; a small volume (0·01–0·02 c.c.) of a 10^{-3} solution of 2268F in saline was then injected into the anterior chamber with a syringe, and the needle was immediately withdrawn to allow observation of the pupil.

Close arterial injections into the tibialis anticus muscle. Cats were anaesthetized with 'veterinary nembutal' (pentobarbital sodium) intraperitoneally (25–30 mg./kg. initial dose). The efficacy of this anaesthetic appears to vary with the age of the stock solution, and it was found that the prescribed dose of 1 gr. /5 lb. body weight, or 26 mg./kg., was usually inadequate, and had to be supplemented by a further 12–30 mg./kg. during the course of the experiment.

The tibialis anticus muscle was prepared for close arterial injection according to the method described by Brown (1938). The sciatic nerve was crushed high up in the thigh by placing a tight ligature round it. The motor fibres to the tibialis anticus were stimulated either (*a*) in the peroneal nerve with shielded silver electrodes, or (*b*) in the sciatic nerve after ligation of its medial branch. For the latter a tubular perspex electrode was buried in the popliteal fossa. Square pulses were led

in from an electronic stimulator in all except one experiment in which a neon discharge was used for stimulation.

Perfusion of the superior cervical ganglion. The experiments were conducted mostly under preliminary ether and then chloralose (75–100 mg./kg.) anaesthesia. Chloralose 'Kuhlmann' was used in most experiments, and chloralose 'Boots' in a few others; nembutal in only two. The cats were of either sex at first, but later only male cats were used.

The preparation of the ganglion was as described by previous workers (Kibjakow, 1933; Feldberg & Gaddum, 1934; MacIntosh, 1938). The diagram in Kibjakow's paper is misleading in several respects, and the reader is referred, for an exact description of the vascular anatomy of this region, to the paper by Davis & Story (1943), which is beautifully illustrated. I am greatly indebted to Dr F. C. MacIntosh for a demonstration of the method. As in his procedure, the post-ganglionic sympathetic trunk is separated, in the final stage of the dissection, from the underlying structures which are then all tied together close to the foramen lacerum posterior. This ligature includes the vagus and accessory nerves (the glossopharyngeal nerve is tied separately), the internal jugular vein, and (usually not seen at this stage but found post-mortem in the latex-injected ganglion) that branch of the occipital artery which divides into the inferior tympanic and the posterior meningeal arteries (Davis & Story, 1943, figs. 1, 2). This is of some importance because the inferior tympanic anastomoses with:—

(*a*) The stylomastoid artery on the same side (Davis & Story, 1943, fig. 2). This branch of the external carotid is given off in the neck (Davis & Story, 1943, fig. 1) slightly above the point where the parent artery is ligated. However, it is probable that the direction of blood flow in this part of the external carotid is reversed after the ligature is tied, i.e. blood would enter the external carotid from the circle of Willis via the arteria anastomotica and the rete externum, and from there it would travel both caudally to the stylomastoid artery, and forward to the nictitating membrane.

(*b*) The intracranial portion of the internal carotid, i.e. that part of it which is now the continuation of the ascending pharyngeal in most cats.

The dissection is greatly facilitated, particularly in its final stages, by retraction of the larynx and pharynx with an illuminated perspex retractor (Coldlite type A 13). This helps to render the nerves visible near their entry into the skull, where the structures lie in a slight recess between the tympanic bulla (which is nibbled away) and the longus colli muscle, which is transected as high as possible.

After completing the dissection, the animals were heparinized with 1000–2500 i.u. Liquemin (Roche), or with a solution made from a Swedish heparin powder kindly presented to the Unit by Dr E. Bárány. The Locke solution for the perfusion, was filtered once or twice through a no. 4 Jena sintered glass filter; a linen filter was interposed between the reservoir and the warming condenser. A static perfusion pressure of 80–200 cm. H_2O was used. The perfusion fluid, after passing through a drop-counting chamber (blood transfusion type of drip), was warmed in a condenser by a hot (39–40° C.) water jacket; the temperature of the perfusion fluid, measured in some of the experiments with a thermo-couple, was 35–39° C. The inflow rate of the perfusion was measured by timing the drops in the chamber with a stopwatch; this was done at frequent intervals. Drugs were injected through a rubber cap into the arterial cannula as close to its tip as possible. It must be pointed out that, nevertheless, doses of drug injected in experiments of this type undergo dilution to an unknown extent by admixture with, and diffusion into, some of the 10 c.c. of perfusion fluid which is contained in the body of the cannula; this could be seen happening with injections of methylene blue. Mixing would occur during the 2–20 sec. latent period (timed with a stopwatch in some of the later experiments) which is observed after the injection of drugs, and its extent would be inversely proportional to the rate of perfusion. The dilution factor will also depend upon the 'dead-space' of fluid within the preparation, i.e. in the common carotid and its ligated branches, and probably affects to a certain extent the sensitivity of the ganglion to drugs. Also, an unknown fraction of the perfusion fluid traverses the superior cervical ganglion, and the rest passes through the nodose ganglion and other nerve trunks, which are also included in the perfusion.

The design of the arterial cannula was slightly modified after the preliminary experiments, and is now as follows: (*a*) it is made of thick glass (*c.* 1 mm. bore), to reduce the dead space and minimize cooling; (*b*) the connexion to the condenser is through a short side-arm near the free end; (*c*) the free end is flanged and carries a well-fitting rubber cap through which the injections are made (for these a no. 12 syringe needle is driven into the very tip of the cannula, into which the needle just fits without sticking); (*d*) the length of the cannula is equal to that of a no. 12 needle.

The venous effluent from the ganglion was collected by cannulating either the internal jugular or, quite frequently when this was too small, the 'back vein' which connects the internal jugular vein, at the level of the ganglion, to the vertebral veins. A convenient form of cannula was made by smoothing the tip of a no. 12 or no. 15 syringe needle, cutting off about 1 cm. of the needle and sealing it into a length (15–20 cm.) of fine bore (0·5 mm.) polythene tubing, which has the advantage of lightness and flexibility. In a few of the experiments cannulation was unsuccessful, but the venous effluent was collected into pads of cotton-wool placed under that vein, or the internal jugular lower down in the neck.

Drug solutions were made up in Locke solution, and were filtered individually and then warmed before injection; the syringes were also warmed. The volume of each injection varied between 0·05 and 0·2 c.c. The acetylcholine solutions were usually made up from a stock solution, 10^{-3} in 5% NaH_2PO_4, but neutral acetylcholine HCl, free from phosphate, was used in later experiments.

The cervical sympathetic preganglionic nerve was not separated from the accompanying vagus, in order to preserve its blood supply. The vago-sympathetic trunk was cut and tied centrally to the ganglion, as low as possible in the neck, and stimulated faradically, at intervals, with shielded electrodes left in position. Contractions of the nictitating membrane were recorded with an isometric lever.

Preganglionic denervation. The right superior cervical ganglion was decentralized in one cat by cutting and avulsing a length of about 1 cm. of the vago-sympathetic trunk as low as possible in the neck, i.e. centrally to the ganglion. The operation was performed under nembutal anaesthesia with the usual aseptic precautions, and time was allowed for the preganglionic fibres to degenerate. The denervated ganglion was prepared for perfusion by the usual procedure 18 days later.

Other experiments on the ganglion. In a few additional experiments the ganglion was exposed, and the point of a fine syringe needle was stuck into it. Small amounts of 2268 F were injected directly into the tissue of the ganglion.

2268 F. Because of its tendency to deliquesce, this substance, a sample of which was kindly supplied to me by Sir Henry Dale, was stored in a desiccator. From this, fresh solutions of the drug were made up for the majority of experiments, but in a few experiments the stock solutions were stored in the refrigerator for several days or weeks without any marked deterioration (see Fig. 8).

The blood supply to the superior cervical ganglion. To ascertain the details of the arterial branches in the ganglion region, and in particular whether the internal carotid is imperforate as it is said to be in most cats, a few of the ganglion preparations were injected at the end of the perfusions with a latex (Neoprene 842 A) containing a dye. The perfused block of tissue was then excised and transferred to 10% formol saline containing 5% glacial acetic acid. In addition, the whole arterial system of the head was injected with latex in several other cats, as follows. The common carotid artery was tied on one side, and the latex was introduced at a pressure of 200–300 mm. Hg through a cannula in the other common carotid artery; this artery was then tied before the cannula was removed, to prevent backflow. The neck was then severed low down and the whole head kept overnight in 10% formol saline containing 5% glacial acetic acid, which sets the latex. Next day the head was skinned and transferred to a 10% formalin solution, and was then dissected.

RESULTS

Frog muscle; rectus abdominis. As stated by Fourneau *et al.* (1944), 2268 F is capable of causing contractions of this muscle. In all of three experiments, two

without and one in the presence of eserine, contractions were obtained with concentrations of 2268F ranging between 10^{-6} and 10^{-5} (see Fig. 1). In two of these experiments the effect of 2268F was abolished by soaking the preparation for 5 min. in 10^{-5} D-tubocurarine; on the other hand, the response to 2268F was not affected by soaking in 10^{-6} atropine for 10 min., or in 10^{-5} for 20 min.

Dorsal muscle of the leech. The leech is a more suitable test for the presence of a nicotinic action than the frog's rectus, since its reaction to acetylcholine, for instance, is unaffected by atropine, and is abolished by nicotine (Chang & Gaddum, 1933; p. 263). Like the frog's rectus, the dorsal muscle of the leech,

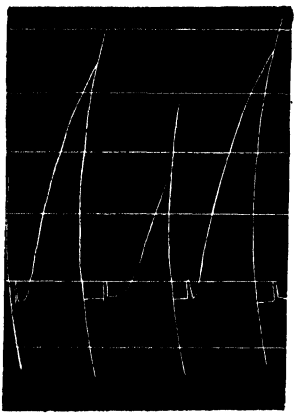

Fig. 1. Effect of 2268F on frog's rectus abdominis muscle. Eserine present (1 in 70,000). Duration of each contraction: 2 min. *A* and *C*, 5×10^{-8} acetylcholine; *B*, 8×10^{-6} 2268F.

not eserinized, responds well to 2268F in concentrations exceeding 10^{-6}. As before, the action of 2268F was abolished by 10^{-5} D-tubocurarine (three experiments); this effect was reversible, but recovery from curarization was very slow (Fig. 2 *C–F*).

Avian iris. The sphincter pupillae in birds consists of skeletal muscle. It has been used in the past as a pharmacological test for drugs possessing nicotinic activity, and 2268F appears to fall into this category. In four experiments on three hens, the injection of 10–20 μg. into the anterior chamber was followed by a prompt meiosis.

Mammalian muscle; tibialis anticus (cats). Close arterial injections of 2268F into the tibialis anticus muscle (10 μg. in two experiments, 20 and 50 μg. in two others) elicit a rapid twitch which is promptly followed (within 40 sec. in Fig. 3) by a long-lasting depression of the response of that muscle both to maximal motor nerve stimuli and to doses of acetylcholine previously effective by close arterial injection. Thus in Fig. 3 an initial injection of 10 μg. of

acetylcholine (at 1) gave rise to a twitch which was equal in height to subsequent twitches elicited by nearly maximal motor nerve stimulation. At 2, the electrical stimulation was interrupted for 1 min., and an injection of 10 μg. of

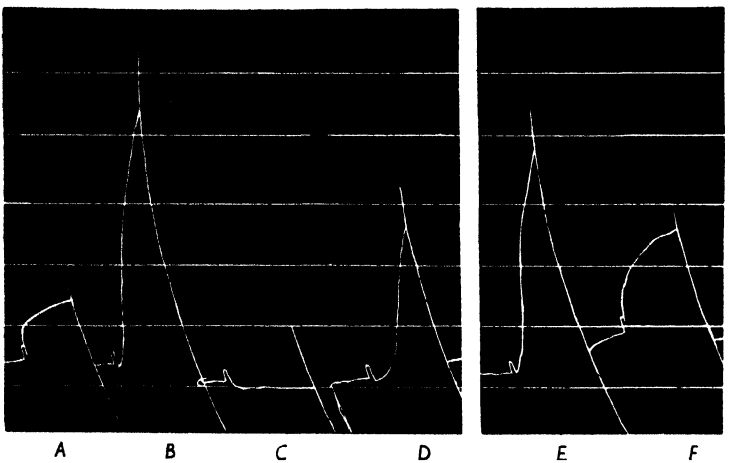

Fig. 2. Contraction of dorsal muscle of leech (not eserinized) by 2268 F, and its abolition by D-tubocurarine. All contractions last for 2·5 min. except for *A*, which is 2 min. *A*, 10^{-6} acetylcholine; *B*, 2×10^{-6} 2268 F. Between *B* and *C* D-tubocurarine chloride (10^{-5}) introduced into bath. *C*, 6 min. later, 2×10^{-6} 2268 F; *D* and *E*, 20 min. and 3 hr. 22 min. later, 2×10^{-6} 2268 F, showing recovery from curarine; *F*, 2×10^{-6} acetylcholine.

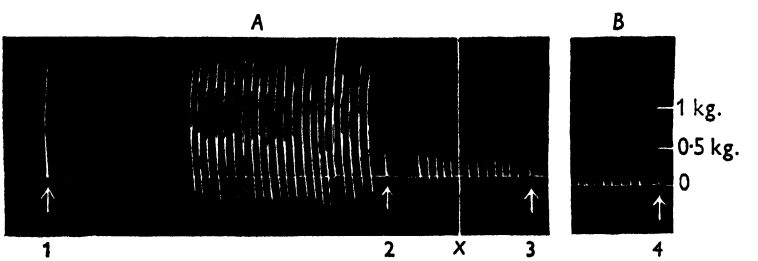

Fig. 3. Cat, 2·3 kg. Tibialis anticus preparation. At 1, initial effect of close arterial injection of 10 μg. of acetylcholine. After this, nearly maximal motor nerve stimulation (frequency 1 in 10 sec.) was started and maintained to the end of the experiment with interruptions for injections at 2, 3 and 4, and for 27 min. between *A* and *B*. Drum stopped at *X* for a few moments. At 2, 10 μg. of 2268 F produce a contraction which is followed by depression of the response to motor nerve stimulation and (at 3 and 4) to close arterial injections of 10 μg. of acetylcholine.

2268 F elicited a small twitch. Stimulation was resumed 40 sec. later, when the depression was already present. After 170 sec. (at 3) the response to a close arterial injection of 10 μg. of acetylcholine was 10% of its original height.

Half an hour later this depression was still present, as shown in Fig. 3*B*, where the responses to maximal motor nerve stimulation and to 10 μg. of acetylcholine (at 4) were smaller still. This prolonged depressive action of 2268F is not unlike that of the stable ester carbaminoylcholine (Bacq & Brown, 1937), and may also be due to the relative stability of this compound.

The twitch produced by 20 μg. of 2268F is shown in Fig. 4. In this experiment a rather higher rate of motor nerve stimulation (every 2 sec. supramaximal) was used, which may account for the slight fatigue shown at the beginning of the tracing. When the response of the muscle had settled down to a steady level, the 2268F was injected (at 3) and this was followed, as before, by profound depression of the response to nerve stimulation and to 10 μg. of acetylcholine given intra-arterially.

In one of these experiments the cat had received 1·4 mg./kg. of atropine sulphate intravenously. The response to 2268 F (and to acetylcholine) was of the usual type.

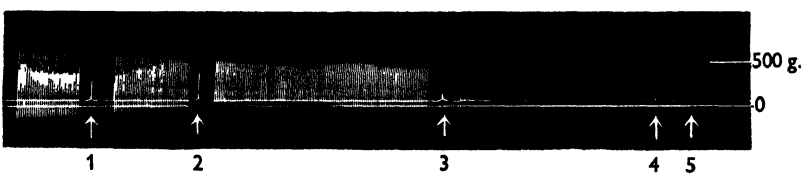

Fig. 4. Cat, 2 kg. Contractions of the tibialis anticus elicited by slightly supramaximal stimulation (3 V.; 1 msec.) of homolateral nerve at a frequency of 1 in 2 sec. Rhythmic stimulation was maintained throughout, with short interruptions at 1–5 for close arterial injection of drugs. At 1, 2, 4 and 5: 10 μg. of acetylcholine; At 3, 20 μg. of 2268 F producing a twitch, followed by profound depression of the response both to motor nerve stimulation and to acetylcholine injections.

The action of 2268F on the superior cervical ganglion; perfusion experiments. In the early experiments of this series, before it was realized how sensitive the superior cervical ganglion was to 2268F, doses of 5–10 μg. of this substance were injected into the perfusion cannula. Within a few seconds of the injection contractions of the nictitating membrane were recorded (Fig. 5). In this range of doses, which elicited maximal effects, 2268F was approximately only twice as powerful as acetylcholine, but its action was rather more prolonged (see also Fig. 8 for a comparison between 1 μg. of 2268F and 10 μg. of A.Ch.). However, the comparison between the two drugs is not really possible in the absence of eserine because acetylcholine is destroyed by cholinesterase on its way through the ganglion, whereas 2268F is not hydrolysed, according to Fourneau *et al.* (1944). One could therefore expect the normal ganglion to be sensitive to much smaller doses of 2268F, and this sensitivity could be expected to be of the same order as, or even greater than, that of the eserinized ganglion to acetylcholine, which is *c.* 0·05–0·2 μg. (Feldberg & Vartiainen, 1934). As shown in Fig. 6, this is so. In this experiment doses of 0·02–0·2 μg. of 2268F had a distinct effect on

the ganglion; 0·01 μg., a perceptible, and 0·005 μg., a just perceptible or threshold effect.

The latency of this effect has been timed more accurately with a stopwatch and compared with that of acetylcholine in a few experiments. Thus, in one

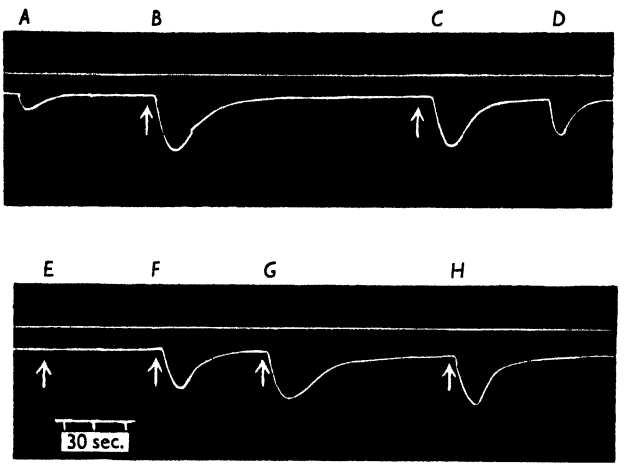

Fig. 5. Cat, 2 7 kg. Effect of 2268 F on perfused superior cervical ganglion. Tension records from homolateral nictitating membrane; a downward movement indicates contraction in this and subsequent records. Top line indicates zero tension in all records. Time in 30 sec. *A* and *D*, response to submaximal preganglionic stimulation of the cervical sympathetic nerve for 5 sec.; *B*, injection of 10 μg. 2268 F; *C*, 20 μg. acetylcholine (containing also 1 mg. of NaH_2PO_4); *E*, control injection of warm saline (0·2 c.c.); *F* and *H*, 10 μg. acetylcholine (containing also 0·5 mg. of NaH_2PO_4); *G*, 5 μg. 2268 F.

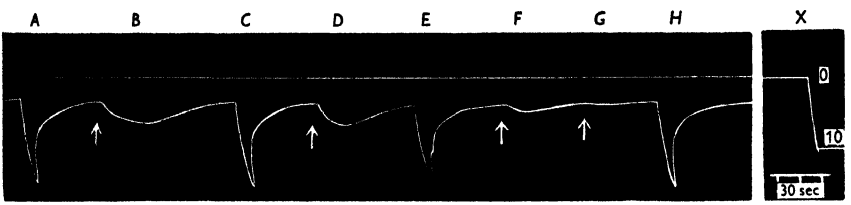

Fig. 6. Cat, 2·75 kg. (sex not noted). Effect of small doses of 2268 F upon the superior cervical ganglion. At *A*, *C*, *E* and *H*, maximal preganglionic stimulation for 5 sec. Injections of 2268 F at *B* (0·1 μg.), D (0·05 μg.), *F* (0·01 μg.) and *G* (0·005 μg.). Calibration (10 g.) shown at *X*.

experiment, the delay from the moment of injection to the beginning of the response was of the order of 9 sec. after 0·5 μg. of 2268 F, and 2–3 sec. after 10 μg. of acetylcholine (A.Ch.). In three other experiments the delays for 2268 F were: (*a*) 15 sec. with doses of 0·02 and 0·05 μg.; (*b*) 6 sec. with 15 μg., and 11 sec. with 5 μg.; and (*c*) 13 sec. with 5 μg. of 2268 F. In all these experiments

the delay with 10–20 μg. of A.Ch. was only 2–3 sec., but in the experiment of Fig. 7 rather longer delays were recorded.

This difference in time lag may be due to differences in molecular weight, configuration, diffusion properties, and anion of the two compounds. The molecular weights of the two are: 2268F (as iodide), 287; acetylcholine chloride, 181·5.

Variations in sensitivity. Towards the end of this series of experiments, variations in sensitivity to 2268F were observed. Whereas previously the ganglion preparations had responded well (in male cats) to 0·005–0·01, 0·02, 0·02, 0·15, 0·02, 0·5 and 0·5 μg. respectively in seven different experiments (and very well to 5 μg. in another (Fig. 5), in a few subsequent experiments the dose of 2268F had to be raised before any response could be obtained. The activity of the 2268F was tested on the guinea-pig's ileum, and an essay against acetylcholine showed that there was no appreciable loss in its effectiveness. The apparent variations in sensitivity in two or three of the male cats could be accounted for by imperfections in the preparations. They were probably due to the development of leakages at one or more of the ligatures, as shown by sudden increases in the perfusion rates during these particular experiments. But the insensitivity which was observed in two other cats, one pregnant and the other pseudo-pregnant, could not be so accounted for.

A critical examination of the superior cervical ganglion preparation and of its suitability as an index of nicotinic action in pharmacological experiments

In pharmacological experiments of this type, one has to be quite certain that the perfusate and drugs injected are, as much as possible, restricted to the ganglion and, what is more important, that there is no direct pathway for any perfusate (and drug) to the nictitating membrane. For it is well known that the smooth muscle in the nictitating membrane is peculiar in that it responds by contraction both to adrenaline and to acetylcholine (and pilocarpine), which elsewhere exert antagonistic effects. Thus it was just conceivable that some of the effects observed above might have been due to a 'muscarinic' action of 2268F directly upon the smooth muscle of the nictitating membrane. These doubts were removed as follows.

A. *Anatomical considerations.* In the majority of the experiments an internal carotid artery was not found when the preparation was made. In only one out of sixteen experiments was a minute blood vessel seen (albeit of venous appearance) in the position of the internal carotid, and this was tied. It was, however, still conceivable that if the internal carotid was so small as to escape notice and ligature, it could provide a hypothetical pathway for the perfusate to reach the nictitating membrane, via the common carotid and internal carotid arteries to the circle of Willis, and then via the rete mirabile externum to the external carotid artery, the infraorbital artery, the arteria angularis, and its branch, the arteria membranae nictitantis (Davis & Story, 1943).

However, Davis & Story (1943) have subjected this region to a very thorough examination; their anatomical findings were based on a careful latex injection technique and on serial sections, which revealed that in ten out of eleven cats the internal carotid artery was imperforate. Below the tympanic bulla, and in its course on to the roof of the bulla, the artery was present only as a vestigial cord without a lumen; only in one cat had it a lumen of 0·2 mm. Moreover, according to the same authors, the distal, intracranial, portion of the internal carotid, which eventually links up with the circle of Willis, has been 'taken over' by, and is functionally the direct continuation of, the ascending pharyngeal artery. The latter was always tied near its origin in the present experiments; and, according to Davis & Story, the direction of blood flow in its distal part is *away* from the circle of Willis. Therefore such a major pathway through the circle of Willis to the nictitating membrane can be excluded. Even if it did exist, the fraction of any dose of drug injected into the common carotid artery, which would eventually reach the nictitating membrane, would be very small, because the blood from the circle of Willis is distributed to several branches, the connexions of the rete are many in number, and so are the branches of the external carotid and angular arteries. Moreover, the entry into, and movement of saline in the internal carotid, when it is present, would be opposed by the arterial pressure of the blood within it, whereas its outflow through the jugular vein is unimpeded. As this point was of some importance in the present series of experiments, the opportunity was taken of examining the arterial pattern of this region, by injecting some of the ganglion preparations with latex, and also by obtaining a complete cast of the intact arterial system of the head in other cats. The results of these experiments fully agree with the detailed description given by Davis & Story. In five out of a total of seven preparations the internal carotid was found arising from a small nipple-like expansion (which was filled with latex) of the common carotid, about 1 mm. caudal to the occipito-pharyngeal trunk. Beyond this expansion the internal carotid could be dissected as a whitish, cord-like structure which adjoined the superior cervical ganglion, and accompanied the untied postganglionic trunk on its lateral side into the carotid foramen. In these five preparations the latex did not enter this cord, although vessels of finer calibre were well filled, for instance the fine arterial twigs to the nodose and superior cervical ganglion; but in the remaining two cats latex *was* found in the internal carotid. Besides this, the latex casts of the ganglion preparations showed the ligatured stumps of the ascending pharyngeal artery, of its ramus m. longus capitis, and of the occipital artery and its branches, of which only those which enter the skull need further description. These are (*a*) a vessel which divides into the inferior tympanic and the posterior meningeal arteries; and (*b*) a minute twig which in *c.* 50 % of cats (Davis & Story) joins the internal carotid nerve and accompanies it to the promontory in the internal ear, where it anastomoses with terminal twigs of

the ascending pharyngeal artery. In the remaining 50% of cats this twig is said to arise from the posterior branch of the inferior tympanic artery. After the usual procedure—which consisted of a separation of the post-ganglionic trunk from the cranial nerves behind it, and then of tying a ligature round the glossopharyngeal nerve and another round the vagus nerve, the accessory nerve, and the internal jugular vein—it was found that the first and the most important of the above-mentioned branches of the occipital was included in the second ligature. The second branch was not seen.

Lastly, there are some minute vessels in the sheath of the postganglionic sympathetic trunk, which have to be left untied for fear of damaging the nerve itself. These might still connect the ganglion-perfusion with the general circulation, but the leakage at this point is probably very small, and, once in the blood stream, drugs would undergo considerable dilution.

The evidence of the simultaneous dilatation of the homolateral pupil. Further evidence is provided by the following observations on the changes in size of the pupil after ganglionic administration of 2268F. When the nictitating membrane retracted there was a dilatation of the homolateral, but not of the contralateral, pupil in six observations in three experiments. In one experiment this effect was timed and it appeared 6 sec. after the moment of injection (dose, 5 μg.). The shortness of this delay suggests a ganglionic action.

It is worth noticing that this pupillary response obtained with 2268F is the reverse of its direct action on the iris, which is a powerful meiosis (Ramanamanjary, 1945; experiments on mice), again suggesting that the perfusate from the ganglion does not reach the blood vessels of the orbit.

C. *Experiments with adrenaline.* It is well known that minute amounts of adrenaline will cause the nictitating membrane to contract and the pupil to dilate, by a direct action. Assuming that adrenaline does not also excite the ganglion cells, then it should be possible to obtain some indication, with it, of the extent to which the ganglion preparation is isolated from the rest of the animal. The injection of even a large dose of adrenaline into the perfusate would not be expected to produce any effect upon the smooth muscles in the orbit unless some of the adrenaline were to escape into the blood stream. The matter was put to the test in two experiments.

In the first, the administration of two doses of 5 μg. of adrenaline to the ganglion did not produce any alteration either in the size of the pupil or in the tension of the nictitating membrane. Yet in the same experiment contractions were recorded from the nictitating membrane (with a delay of 17 and 11 sec.), and the pupil was observed to dilate (after 6 sec.), after 5 and 10 μg. of 2268F.

In the second (Fig. 8), injections of 5 and 10 μg. of adrenaline were again ineffective, yet with 0·5 and 1 μg. of 2268F the nictitating membrane retracted repeatedly.

Thus in both these experiments the ganglion seems to have been reasonably isolated from the rest of the circulation. Moreover, neoprene casts were made of these two preparations at the end of the perfusions, and showed that the internal carotid was imperforate in both. One is therefore driven to the conclusion that the observed effects of 2268F were purely local in origin, i.e. ganglionic.

Brief mention must be made of an adrenaline after-effect which occurred in the second experiment, and which is visible in Fig. 8. For more than 18 min. after the second dose of adrenaline (10 μg.) the ganglion did not respond to 1 μg. of 2268F, although it did respond to 10 μg. A.Ch. as before. But after 56 min. the ganglion response to 2268F returned and remained until it was abolished by nicotine. In this experiment, too, there appeared to be a dissociation of the pupillary and nictitating responses: after injections of 2268F (1 μg.), and of A.Ch. (10 μg.), there was no dilatation of the pupil, although maximal preganglionic stimulation did dilate the pupil widely. This dissociation of the two effects was present at the very beginning of the experiment, i.e. before the administration of adrenaline. A possible explanation of this unusual effect may be found in the description given by Langley (1904, p. 251) of an occasional variation, found in some cats, in the location of the pupillo-dilator sympathetic ganglion-cells. These may sometimes be situated *outside* the superior cervical ganglion, on the course of the cervical sympathetic, proximal to the ganglion.

D. *Experiments with atropine intravenously.* The hypothetical muscarinic effect on the nictitating membrane of any 2268F which might escape out of the perfused ganglion into the general circulation can be eliminated by the administration of atropine to the whole cat after the perfusion has begun. For this experiment to be successful, it is important to ensure that blood does not enter the ganglion from the atropinized circulation via anastomoses, by raising the perfusion pressure until the venous effluent from the ganglion is quite blood-free. If this precaution is taken, then any effect produced by injections of 2268F into the ganglion perfusion can only be due to stimulation of the ganglion cells. But it must be pointed out that in about 50% of cats a proportion of the post-ganglionic nerve fibres to the nictitating membrane are cholinergic, and that, in these, atropine will reduce both the effect of nervous stimulation (Bacq & Fredericq, 1935; Secker, 1937) and of pharmacological agents activating the ganglion. In such a case the experiments proposed in this section would be inconclusive. This was so in two experiments. However, it was conclusive in two other experiments, which will be described in detail.

The first of these is illustrated in Fig. 7. Brisk responses of the nictitating membrane were obtained with 5 and 10 μg. of 2268F administered to the ganglion on the right side. The cat was then given 2 mg. of atropine sulphate intravenously; 2 min. later the right pupil was dilated and the tissues in that orbit could be assumed to be under the influence of atropine. A minute later 10 μg. of 2268F, injected into the ganglion perfusate, produced approximately the same response as before (Fig. 7, 4). This effect was considerably reduced by the injection into the ganglion perfusate of a total of 13 μg., and later it was completely abolished (temporarily) by a further 10 μg. of C_6 (Fig. 7, 10)—i.e. *bis*trimethylammonium hexane diiodide. It is known that this compound has

a curare-like effect on ganglionic synapses (Organe, Paton & Zaimis, 1949). After 25 min. the ganglion had recovered from the C_6 and was responding again both to preganglionic stimulation, to 10 μg. 2268F, and to 10 μg. of A.Ch. Atropine (0·2 μg.) was then injected into the ganglion perfusate, and this abolished the effect of 10 μg. 2268F (Fig. 7, 14), but not of 10 μg. of A.Ch.

A similar result was obtained in a second experiment which is illustrated in Fig. 8, and which has already been partly described. Here also 2 mg. and later a further 3 mg. of atropine, administered intravenously to the rest of the cat, did not abolish the effect of 1 μg. of 2268F, although the presence of a mydriasis on the homolateral side showed that the atropine had reached the orbit con-

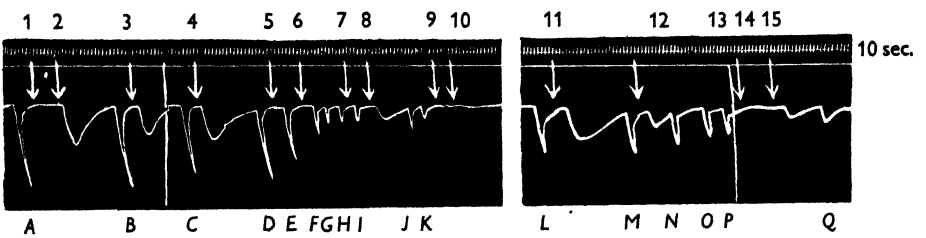

Fig. 7. Cat, 3·4 kg. (♂ castrate). Perfusion of right superior cervica ganglion. *A–Q*, maxima preganglionic stimulation for 5 sec. At 1: 2 μg. of 2268F; no response. At 2: effect of 10 μg. (delay timed on stopwatch: 7 sec.), and at 3, of 5 μg. of 2268F (delay: 11 sec.). Between 3 and *C*, drum stopped and 2 mg. of atropine sulphate administered to the cat intravenously at time 2.56. At 2.58 right pupil was dilated. *C* shows a slight decrease in the response to preganglionic stimulation. 4, at 2.59: 10 μg. of 2268F (delay 10 sec.); 5, at 3.02: 0·3 μg. of C_6; 6, at 3.03: 5 μg. of C_6; 7, at 3.05½: 8 μg. of C_6; 8, at 3.06½: 10 μg. of 2268F; 9, at 3.14: 10 μg. of C_6; 10, at 3.15: 10 μg. of 2268F, response abolished; 11, at 3.40: 10 μg. of 2268F, response fully recovered (delay: 11·5 sec.); 12, at 3.44: 10 μg. of A.Ch. (delay: 7·5 sec.); 13, at 3.48 (after P) 0·2 μg. atropine into the ganglion perfusion; 14, at 3.49: 10 μg. of 2268F: (effect abolished); 15, at 3.50: 10 μg. of A.Ch. (delay: 12 sec.).

cerned. In contrast, the injection of 0·2 μg. of atropine into the ganglion perfusate promptly abolished the response to 2268F, as did, earlier in the experiment, paralysis of the ganglion cells by 100 μg. of nicotine.

The antagonism of 2268F by the administration of atropine to the ganglion. The last observation shows that the stimulating action of 2268F on the ganglion is antagonized by very small amounts of atropine sulphate. This was found in four other perfusion experiments, which will be considered in detail below. The intraganglionic dose of atropine required to abolish the effect of 2268F is so small that it would be unlikely, even in the event of a small proportion of it reaching the general circulation, to atropinize the nictitating membrane. The efficacy of 0·2–1 μg. of atropine in antagonizing the action of 2268F thus bespeaks a ganglionic effect.

In the first experiment 0·5 μg. of 2268F and 10 μg. of A.Ch. (neutral, i.e. free of phosphate) elicited repeatable responses which were equivalent. Atropine

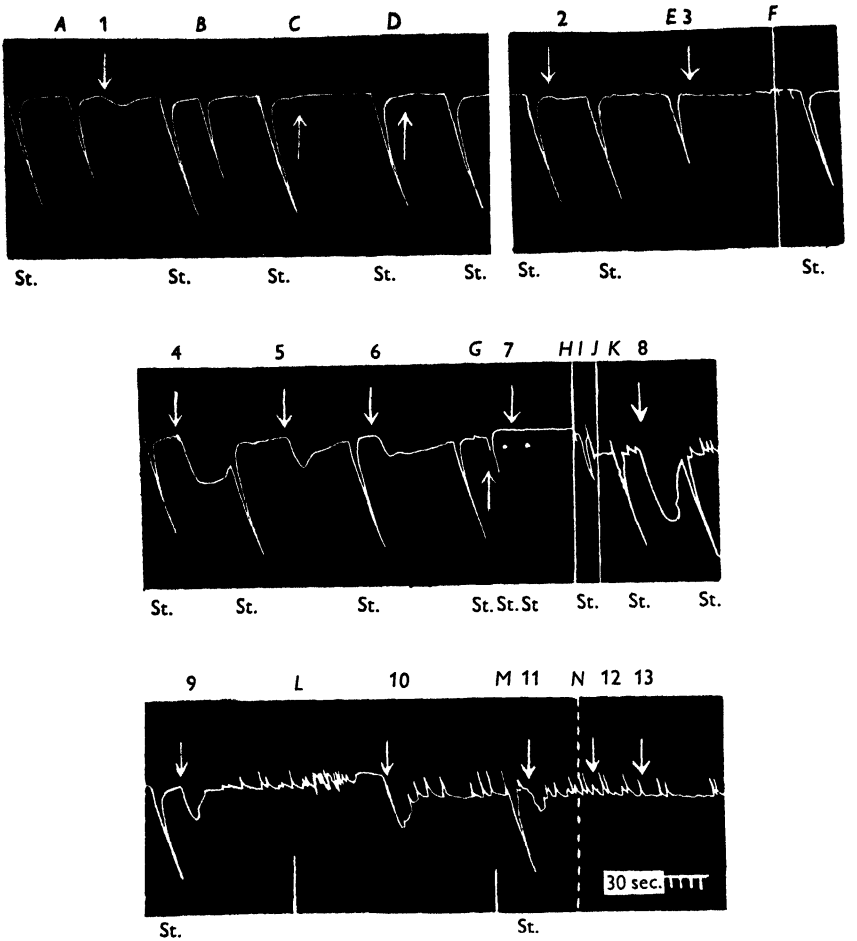

Fig. 8. Cat, 3·7 kg. (♂ castrate). Perfusion of ganglion. Maximal preganglionic stimulation (duration 5 sec.) is labelled 'St' throughout. At *A*, *B*, *and E*: 10 μg. A.Ch. At *C*, 5 μg. and, at *D*, 10 μg. of adrenaline have no effect. The numbers 1–13 refer to injections of 2268 F. The solution injected at 1 and 3–5 was 3 weeks old; a freshly made solution was used for the remaining injections. At 1: 0·5 μg. At 2 (11½ min. after *D*): 1 μg.; the absence of any response is probably an after effect of the adrenaline. At 3 (18 min. after *D*): 1 μg.; still no response. *At F, drum stopped for* 31 *min.* At 4 (56 min. after *D*): 1 μg. (old) is now effective; the inhibition has worn off. At 5, 1 μg. (old) and at 6, 1 μg. (new): the two solutions are about equally active. At *G*, 100 μg. of nicotine hydrogen tartrate: 30 and 90 sec. later there is no response to preganglionic stimulation (marked by the 2 white dots), due to paralysis of ganglion cells. At 7 (60 sec. after *G*): 1 μg. 2268 F has no effect. *Drum stopped at H and J.* At *I* (7½ min. after the nicotine) and *K* (15½ min. after it): maximal preganglionic stimulation, showing the return of transmission. At 8: 1 μg. 2268 F is again effective. From now on (8 hr. from the induction of anaesthesia) the tracing is marred by the appearance of spontaneous movements of the eyeball, but the usual responses may be discerned superimposed upon these. At 9: 1 μg. 2268 F. At *L*, 2 mg. atropine intravenously: the pupils dilate. At 10 (3 min. later): 1 μg. of 2268 F. At *M*, another 3 mg. atropine intravenously. At 11: 1 μg. of 2268 F. At *N*, 0·2 μg. of atropine into the ganglion perfusion. At 12 (½ min. later) and 13 (2 min.): 1 μg. of 2268 F has no effect.

0·2 μg. abolished the response to 0·5 μg. of 2268F administered 2½ and 6½ min. later, but did not alter the response to 10 μg. of A.Ch., given 4 min. after the atropine, or to preganglionic stimulation at intervening times. After 48 min. the ganglion had recovered fully from the atropine and gave again its original response to 0·5 μg. of 2268F.

In the second, in which there was some leakage of fluid from the perfused mass of tissue, 0·5 μg. of atropine almost abolished the response to 1 μg. of 2268F, but did not alter the equivalent effect of 20 μg. A.Ch. (neutral). After 8½ min. the response to 1 μg. of 2268F had recovered to about 70% of its previous height. After a further 1 μg. of atropine, it was again abolished, and the response to 20 μg. of A.Ch. was reduced by about 20%, although the effect of preganglionic stimulation was unaltered. After 38 min. the response to 1 μg. of 2268F was fully restored but was again abolished by 1 μg. of atropine.

In the third, 1 μg. of atropine abolished the effect of 0·15 μg. of 2268F and reduced the effect of 10 μg. of A.Ch., but did not alter the response to preganglionic stimulation.

In the fourth, 20 μg. of atropine sulphate abolished the effect of 5 μg. of 2268F and of 10 μg. of A.Ch.

The ganglionic action of 2268F is still present after preganglionic denervation. There still remained, at this stage of the investigation, the hypothetical possibility that the excitation of ganglion cells by 2268F could arise indirectly as a result, for example, of a primary stimulation of the preganglionic nerve endings within the ganglion. Such an action would equally well be blocked, at the next link in the chain of events, by nicotine and C_6, though not by small doses of atropine. It was possible to eliminate this alternative by examining the effect of 2268F on a ganglion of which the preganglionic nerve supply had been cut and allowed to degenerate. This ganglion was perfused 18 days after preganglionic denervation. As was to be expected, the preparation was abnormally sensitive to acetylcholine, and a standard dose of 0·05 μg. was adopted throughout the experiment (Fig. 9). The ganglion also responded to 0·05, 0·1, 0·5 and 1μg. of 2268F (solution 29 days old). As the effect of 1 μg. was smaller than that of 0·5 μg. (which suggested a curarizing action), the latter was adopted as the standard dose of 2268F for the rest of the experiment. The delay times recorded with this were 7–8 sec. (three measurements).

Paralysis by nicotine and atropine. 20 μg. of nicotine produced a stimulation of the denervated ganglion (with a delay of 1–2 sec. from the moment of injection) followed by paralysis. Two, and four minutes later, the standard doses of A.Ch., and, in between, the standard dose of 2268F, were all ineffective. The paralysis was reversible, and, as the nicotine was washed out, the ganglion recovered and responded to A.Ch. 32 min., and to 2268F, 36 min. later. An hour later this observation was repeated, and the result, which was identical, is shown in

Fig. 3 of the next paper; the delay time for the second dose of nicotine, at *I*, was 2–3 sec.

As in the other experiments described above, the ganglionic action of 2268F (and of other drugs discussed in the next paper) was reversibly abolished (Fig. 9*G*) by 0·2 μg. of atropine administered to the ganglion 90 sec. earlier at *F*; the recovery of this response is shown 15 min. later at *M*. The action of A.Ch. was still present during this period (at *H*, 2½ min. after the atropine), but it was reduced as in some of the previous experiments. Also, at the conclusion of this experiment 1 hr. later, the intravenous administration of 3 mg. of atropine to the rest of the cat did not alter the ganglionic response to 2268F (Fig. 3*R* of the next paper). A surprising result was obtained with adrenaline; 10 μg. of an old solution (29 days, slightly pink) gave rise to a small response after 20 sec.,

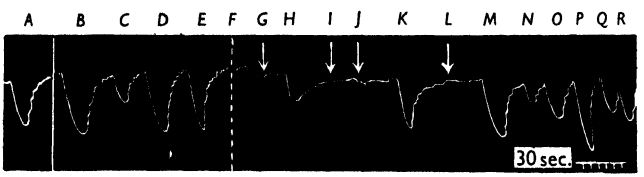

Fig. 9. Cat, 3·2 kg. (♂). Perfusion of the superior cervical ganglion, 18 *days after preganglionic denervation;* showing the presence of a response to 2268F, its abolition by 0·2 μg. of atropine, and subsequent recovery. At *A*, *J*, *L* and *N*, 50 μg. $BaCl_2.2H_2O$. Between *A* and *B*, an interval of 9 min. At *B*, *D*, *G* and *M*, 0·5 μg. of 2268F. At *C* and *I*, 0·5 μg. of pilocarpine. At *E*, *H*, *K* and *P*, 0·05 μg. of acetylcholine. At *F*, 0·2 μg. of atropine into the ganglion perfusion, followed by abolition (with recovery later on) of the response to 2268F, pilocarpine and barium, and reduction in the response to A.Ch. At *O*, *Q* and *R*, 200 μg. $BaCl_2.2H_2O$.

but, 6½ min. later, 20 μg. of a fresh solution had no effect upon the ganglion and did not alter the response, 2 and 4 min. later, to A.Ch. and 2268F.

Other experiments with 2268*F*. In a few experiments the ganglion was stabbed with a fine needle. When the transient effect of the stab had worn off, 2268F was injected through the needle into the substance of the ganglion in doses of 0·2, 0·5, 1, 5 and 50 μg., all of which produced effects. In experiments of this type it was unlikely that any drug injected intraganglionically could reach the nictitating membrane except after a considerable delay. However, this procedure has the disadvantage that when the ganglion has been punctured more than once further injections leak out of the stab-holes, and do not produce any effect.

Lastly, one of the earlier perfusion experiments is included in this section because it was performed on a dead cat. The animal died just before the perfusion was begun; nevertheless, it yielded useful information. In this experiment there could, again, be no question of any injected substances reaching the nictitating membrane by the blood stream, as the circulation was

arrested. Yet the usual effect of 2268F was observed with 100 μg. This, being a very large dose, was followed by signs of curarization from which the ganglion recovered 12 min. later. At the end of this experiment, when the preparation ceased to function, 0·5 c.c. of a filtered methylene blue solution (0·25%) was injected into the perfusion cannula. Within 30 sec. the superior cervical ganglion and postganglionic trunk were intensely stained as far as the foramina in the skull, but the nictitating membrane was equally pale on both sides.

DISCUSSION

The results show, first, that 2268F, like its homologue 2249F, is capable of stimulating ganglion cells. But, because of the greater sensitivity to small doses of atropine, this ganglionic action is likely to be missed in experiments of the type performed by Fourneau *et al.* (1944). These authors used the presence of a pressor response in 'fully atropinized dogs' (dose of atropine not stated) as their criterion for nicotinic activity. With regard to this criterion the following comment must be made: whereas it is certain that nicotine itself raises the blood pressure of an atropinized animal, yet this test is not an infallible criterion of nicotinic activity in the widest sense. This term has come to mean, in pharmacology, a stimulating action on ganglion and other nerve cells (and also on end-plates). Now it is quite certain that acetylcholine is endowed with nicotinic activity, yet, although it usually does display a pressor action after atropine, it does not do so infallibly (Linegar, Herwick & Koppanyi, 1939); vasodepressor responses may be obtained with certain doses of atropine and acetylcholine. Moreover, some of the nicotinic effects of acetylcholine on the central nervous system appear to be sensitive to atropine (numerous instances quoted in the review by Feldberg, 1945).

Therefore, a more satisfactory criterion for a nicotinic action would appear to be the ability to excite the ganglion cells of a relatively isolated preparation such as that of the superior cervical ganglion. As regards 2268F, the present experiments have shown that this criterion is satisfied, and in several experiments with doses (0·005, 0·01, 0·02 μg.) which are comparable, for instance, to those used by Emmelin & Feldberg (1947) in their experiments on the gut, i.e. 0·025–0·1 μg. in a 10 c.c. organ bath. Therefore the assumption which was made throughout their paper, that the effects of 2268F (and also of choline and pilocarpine, which are both endowed with nicotinic activity) were restricted entirely to the smooth muscle fibres, is unjustified. Their own tracings show that the response of the gut to 2268F is reduced in size after nicotine-paralysis of the ganglion cells, and enhanced by eserine. The conclusions deduced from their experiments appear to be equally unjustified; these are discussed at greater length in the appendix to the next paper.

One further point emerges from these results, namely that in 2268F we have an example of a substance which is antagonized by atropine in most of its

actions yet, nevertheless, is a potent stimulator of ganglion cells. In this it may be representative of a category of drugs, of which the ganglionic action has not yet been discovered because, so far, only the effect on the blood pressure has been used as the criterion of nicotinic activity. As we have seen with 2268F, the ganglionic action would be masked throughout in experiments of that type, first by the powerful depressor or muscarinic action, and then by the presence of atropine. One cannot help wondering whether perhaps natural muscarine might not fall into such a category. Its action has not so far been examined upon an isolated ganglion preparation such as the superior cervical. But the fact that it is a quarternary ammonium compound and probably quite close in structure to 2268F and 2249F, both of which are endowed with a ganglion-stimulating activity, speaks in favour of such a possibility. Such a finding would be in agreement with the observation of Feldberg, Minz & Tsudzimura (1934) that natural muscarine (and also pilocarpine) brings about a release of adrenaline from the suprarenal medulla—an action which has been classed as nicotinic, but which, they also found, was abolished by atropine. It is also interesting to note that anhydro-muscarine (betaine aldehyde) is endowed with nicotinic properties (Voet, 1930). Lastly, it would appear that natural muscarine may affect the central nervous system. Thus it is stated by Sollmann (1943) that muscarine causes acute excitement and violent delirium. The ingestion of *Amanita muscaria*, which is used as an intoxicant in certain parts of the world, produces the same effects (which are well described in a short story by H. G. Wells, *The Purple Pileus*).

For the detection of an action upon the superior cervical ganglion it is absolutely essential that the sample of natural muscarine should be of a high degree of purity, because crude extracts of *Amanita muscaria* may contain more choline than muscarine (20:1; King, 1922). As it is known that choline stimulates the cells of the superior cervical ganglion (Feldberg & Vartiainen, 1934), its presence in the extract would lead to confusion. At the moment a supply of purified natural muscarine is unfortunately not available.

SUMMARY

1. Experiments are described, which show that 2268F has a number of nicotine-like actions.

2. Thus it contracts the frog's rectus abdominis and the dorsal muscle of the leech, and these effects are abolished by D-tubocurarine. It also contracts the sphincter pupillae of the hen's eye, which is a nicotinic action.

3. Close arterial injections of 10–50 μg. elicit a distinct twitch from the tibialis anticus muscle of the cat, followed by a prolonged curarizing action, during which the effect of maximal nerve stimulation and of close arterial injection of acetylcholine are both depressed.

4. Small doses of 2268F stimulate the superior cervical ganglion. This action is abolished by nicotine, by C_6 (hexamethonium diiodide) and by very small amounts of atropine (e.g. 0·2 μg.). It was present in a preganglionically denervated preparation.

My thanks are due to Miss Jean Barrett for her assistance, to Dr W. D. M. Paton for a gift of C_6; and to Dr A. O. Zupancic and Mrs G. Leydhecker for their help in some of the experiments. The neoprene was kindly presented by Messrs Durham Raw Materials Ltd.

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THE NICOTINIC ACTION OF SUBSTANCES SUPPOSED TO BE PURELY SMOOTH-MUSCLE STIMULATING. (B) EFFECT OF $BaCl_2$ AND PILOCARPINE ON THE SUPERIOR CERVICAL GANGLION

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This paper is concerned with the ganglionic actions of two substances ($BaCl_2$ and pilocarpine) which are usually assumed to be purely smooth-muscle stimulating. It was pointed out previously (Ambache, 1946) that a number of drugs may owe at least part of their action on the gut to an indirect effect upon the intestinal innervation. Some of the substances used in the experiments described in that paper were known to affect nerve cells and their fibres or endings in other parts of the body. For instance, KCl releases acetylcholine from a wide variety of cholinergic nerve endings, and stimulates ganglion cells and nerve fibres as well. It was known, also, that $BaCl_2$ exerts a veratrine-like action on motor nerve fibres and endings in the toad (Dun & Feng, 1940), and Feng (1937) has suggested, further, that barium may induce a leakage of acetylcholine at motor endings. Lastly, it had been shown by Chou & Chin (1943) that minute amounts of $BaCl_2$ (0·0001–0·0018 mM/kg.), introduced into the cerebrospinal fluid, give rise to tetanic spasms and to convulsive seizures as a result of stimulation of the central nervous system. In agreement with all this, evidence was produced (Ambache, 1946) to show that both these substances, and possibly histamine too (which is capable of stimulating certain types of sensory nerve endings in the skin, and of eliciting axon reflexes) could not be regarded as purely smooth-muscle stimulating substances.

In repeating some of these experiments, Emmelin & Feldberg (1947) used three drugs, in crucial 'control' experiments, on the assumption that they were purely smooth-muscle stimulating substances, namely, choline, pilocarpine, and 2268F. These drugs were believed to be free from any action on the ganglion cells. Their argument was largely based upon this assumption, which for 2268F

has been disproved in the preceding paper, and for choline and pilocarpine was anyway incorrect. For it was well known, and from evidence provided by one of these authors, that choline stimulates ganglion cells in small doses, e.g. 25 μg. injected into the perfusion system of the superior cervical ganglion (Feldberg & Vartiainen, 1934); the dose used by Emmelin & Feldberg (1947) was 300 μg. into a 10 c.c. organ bath. Dale & Laidlaw (1912) had already shown that pilocarpine can stimulate the superior cervical ganglion when applied externally. Later, Bacq & Simonart (1938) described the nicotinic action of pilocarpine on the blood pressure; and Marrazzi (1939) found electrical evidence of an increase in ganglionic activity produced by the same drug, an effect which was antagonized by atropine. Lastly, an analogous action of pilocarpine upon the suprarenal medulla had been recognized previously by Feldberg, Minz & Tsudzimura (1934), who found that pilocarpine brought about a release of adrenaline which could be abolished by atropine.

In the course of the perfusion experiments described in the previous paper, the opportunity was taken to verify the fact that pilocarpine can stimulate the superior cervical ganglion, and it has also been found that $BaCl_2$ does the same. Since it shows that $BaCl_2$ is capable of exciting nerve cells, this finding confirms the point of view taken previously that the action of $BaCl_2$ on the gut is, at least in part, due to a stimulation of the motor innervation within it.

The method is the same as in the preceding paper and needs no further description.

RESULTS

Pilocarpine.

The observations of Dale & Laidlaw (1912), and of Marrazzi (1939), were confirmed in three different experiments, with doses of pilocarpine ranging from 0·25 to 10 μg. One of these is illustrated in Fig. 1, which is the continuation of the experiment described in Fig. 6 of the preceding paper.

Denervated ganglion. The action of pilocarpine was present in a ganglion 18 days after preganglionic denervation, and is shown in Fig. 9*C* of the preceding paper. The threshold dose in this particular experiment lay between 0·1 and 0·5 μg., and the latency of the response shown in Fig. 9*C* was 6 sec. The effect of the same dose of pilocarpine was abolished, at *I*, by 0·2 μg. of atropine administered to the ganglion 5 min. earlier.

$BaCl_2$.

It has been found that $BaCl_2.2H_2O$, injected into the perfusion system in doses ranging from 50 to 500 μg., exerts a definite stimulating action upon the ganglion. In all of four experiments 200 μg. (and in a fifth, 500 μg.) of $BaCl_2.2H_2O$ produced marked contractions of the nictitating membrane after the usual latent period inherent in the perfusion system.

It was noticed in one of these experiments (that of Fig. 2) that the pupil dilated simultaneously on the homolateral, but not on the contralateral, side. In this experiment also, a paralytic effect was observed after a second dose of $BaCl_2.2H_2O$ (100 μg. at *D* in Fig. 2). This was administered 5 min. later and elicited a slightly smaller, but equally prolonged, response, which was followed by a reduction in the response to preganglionic stimulation 4 min. later, to $\frac{1}{4}$ of

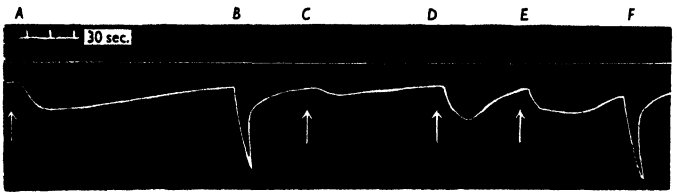

Fig. 1. (continuation of Fig. 6 of preceding paper). Effect of pilocarpine upon the superior cervical ganglion. At *B* and *F*, maximal preganglionic stimulation for 5 sec. Injections of pilocarpine nitrate at *A*, 1 μg.; at *C*, 0·25 μg. *D* shows effect of 10 μg. of acetylcholine (containing also 0·5 mg. of NaH_2PO_4); and *E*, of 0·2 μg. of 2268 F.

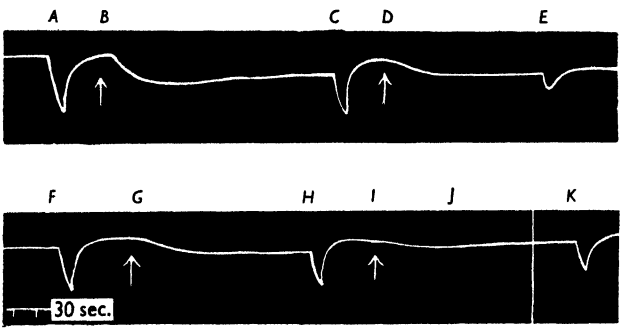

Fig. 2. Cat, 2·75 kg., pregnant. Effect of $BaCl_2$ on superior cervical ganglion by perfusion. At *A*, *C*, *E*, *F*, *H*, *J*, and *K*, maximal preganglionic stimulation; at *B* and *I*, injections of 200 μg. of $BaCl_2$; at *D*, 100 μg. $BaCl_2$; at *G*, 50 μg. $BaCl_2$. The drum was stopped between *J* and *K*; the actual time interval between them was 7 min.

its previous size. After a further 3 min. the response to preganglionic stimulation had recovered to $\frac{3}{4}$ of its original size. Later, 200 μg. of $BaCl_2.2H_2O$ (at *I*) had a much smaller stimulant effect upon the ganglion, and was followed by an absence of response to preganglionic stimulation at *J*, and its restoration 7 min. later at *K*.

In another of these experiments the effect of 500 μg. of $BaCl_2.2H_2O$ was reduced by 50 % after a dose of 1 μg. of atropine administered to the ganglion $2\frac{1}{2}$ min. previously.

Denervated ganglion. As shown in Fig. 3 below, and in Fig. 9 of the preceding paper, the denervated ganglion responded to doses of $BaCl_2.2H_2O$ ranging

from 50 μg. to 1 mg. This action was reversibly abolished by nicotine (Fig. 3 *L* and *M*, with recovery at *Q*), and by atropine (Fig. 9 of preceding paper, *J* and *L*). The latency of the response was 8, 7·5 and 13 sec. for three doses of 50 μg., 9 sec. for 200 μg., and 8 and 5 sec. for two consecutive doses of 1 mg. (at Fig. 3*E* and *F*). The effect of the second dose of 1 mg., at *F*, was smaller than that of the first at *E*.

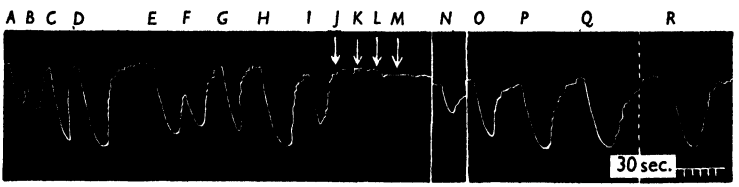

Fig. 3. Cat, 3·2 kg. (♂). Perfusion of the superior cervical ganglion decentralized 18 days previously. Continuation of Fig. 9 of the preceding paper. Showing ganglionic effects of barium and their abolition by nicotine. Injections of $BaCl_2.2H_2O$: at *A*, *L*, and *Q*, 200 μg.; at *B*, 250 μg.; at *E*, *F*, and *M*, 1 mg. Injections of 0·05 μg. acetylcholine at *C*, *G*, *J*, *N*, and *O*. Injections of 0·5 μg. 2268F at *D*, *H*, *K*, *P*, and *R*. 20 μg. of nicotine hydrogen tartrate at *I*, followed by paralysis of the responses to A.Ch. (*J*), 2268F (*K*), and barium (*L*, 200 μg., and *M*, 1 mg.), with recovery of all three later at *O*, *P*, and *Q*. Between *Q* and *R*, 3 mg. of atropine intravenously produced dilatation of the pupil but did not affect the response to 2268F at *R*, 2 min. later. Drum stopped 6 min. between *M* and *N*, and 5 min. between *N* and *O*.

DISCUSSION

The present findings provide direct evidence that $BaCl_2$ has, as postulated previously, an action on nervous tissue.

The experiments on the denervated ganglion show that the action of barium is still present after the preganglionic nerve endings have degenerated, and the fact that this action is abolished by nicotine suggests that the effects observed were due to a stimulation of the ganglion cells proper. These findings confirm the interpretation of the mode of action of barium on the gut which was put forward in an earlier paper. This point of view now receives further support from the recent observation of Collins (1948), who finds that the effect of $BaCl_2$ on the gut is depressed (in four out of five experiments) by the tetra-ethyl-ammonium ion, which is a ganglionic poison. Also, if the earlier work of Berkson (1933*a*, *b*) on the electrical activity of the isolated gut is consulted, one is struck by the correspondence between the type of electrical change immediately consequent upon the administration of $BaCl_2$ (Berkson, 1933*a*; Fig. 2*C*), and that seen after nicotine (Berkson, 1933*b*; Fig. 3*A*). The responses are identical and quite unlike anything else shown in his records.

A curarizing effect of $BaCl_2$ is shown in Figs. 2 and 3F, and may account for the variability in its action on the gut found by Emmelin & Feldberg (1947, fig. 7*A*), particularly if the $BaCl_2$ is administered repeatedly at short intervals,

as in their experiments. Also, it is known clinically that in barium poisoning, which occurs in some parts of China, and is known as Paping, 'occasionally the limbs are found to be spastic, but, when this is overcome, they remain flaccid, or complete paralysis is present' (Allen, 1943, p. 248).

It has also been reported by Thienes (1927) that $BaCl_2$ (1:100,000 to 1:20,000) elicited, in seven out of 100 experiments, *inhibitory* responses in the rabbit gut, an effect which it might be easier to explain on the basis of an action on the innervation of the gut rather than directly on the muscle fibres.

Lastly, the fact, which is illustrated in Fig. 9 of the preceding paper, that the ganglion stimulating action of barium may be sensitive to small doses of atropine, could perhaps explain some of Emmelin & Feldberg's findings with these two drugs in combination. They state that in some of their experiments, for example that of fig. 7 *b* of their paper, the barium contractions were definitely reduced by atropine. Moreover, in other experiments, the barium contractions were not restored, when, after washing the atropine out, the muscle was again sensitive to acetylcholine. As shown above, in Fig. 9 of the preceding paper, the ganglionic response to A.Ch. (at *K*) may recover before that of barium (at *L* and *N*), suggesting a slower rate of recovery for barium after atropine, which may explain their findings.

SUMMARY

1. The existence of a ganglion-stimulating action of pilocarpine (Dale & Laidlaw, 1912) has been verified. It was present in a preganglionically denervated ganglion and was abolished by atropine.

2. $BaCl_2$ is capable of stimulating the superior cervical ganglion. This action was present in a decentralised ganglion, and was reversibly abolished both by nicotine and by atropine.

Note added in proof. The author's attention has been kindly drawn by Prof. J. H. Burn to a thesis written in his department by Dr T. C. Chou (1947), which includes a description of some experiments with barium on the perfused superior cervical ganglion. Special mention must be made of the two experiments illustrated in the figures, which show that the same dose of $BaCl_2$ may produce stimulation of one ganglion, and depression of another—a result somewhat analogous to that shown in Fig. 2 above (*B* and *I*), obtained in one and the same ganglion.

I wish to thank Miss Sheila Stennett for her assistance.

APPENDIX

In this appendix it is proposed to discuss the relevance of the findings described in this and the preceding paper to present-day conceptions of drug action on smooth muscle. For the sake of clarity, it is necessary to summarize as briefly as possible both my previous results (1946) and those of Emmelin & Feldberg (1947).

First, it had been found by Dikshit (1938) that cooling abolishes the production of acetylcholine in the gut. This method was therefore used by the

present author as a means of 'denervating' the gut. After cooling, it was found that the response to doses of KCl and $BaCl_2$, which had been previously effective, was considerably reduced, and later absent. This change was attributed to a functional 'denervation' produced by the cooling, and was taken as confirming the hypothesis that these substances must owe at least part of their effect to an action on the nervous apparatus in the gut. As regards the effect with $BaCl_2$, these results were confirmed by Emmelin & Feldberg, who also found that the sensitivity of the gut to KCl decreased (at first) to a greater extent than to acetylcholine.

Use of pilocarpine in cooling experiments. In controlling these particular experiments, Emmelin & Feldberg used pilocarpine, and they found that cooling diminished the response of the gut to pilocarpine to a much greater extent than was expected. Because of this, they concluded that the principal effect of cooling was due to a depression of the muscle fibres themselves since, it was argued, pilocarpine was a 'purely smooth-muscle stimulating substance'. However, the use of pilocarpine provides no control; it is, as we have seen, as though one were to use nicotine itself, because pilocarpine possesses a nicotinic action (Dale & Laidlaw, 1912). This is also shown by the present experiments on the ganglion, in which 0·25–1 μg. of pilocarpine had distinct effects on the ganglion; the dose used by Emmelin & Feldberg was 2·5 and 10 μg. Now, the action of nicotine on the gut is reduced by cooling, and then abolished (Vogt, 1943); and that component in the response to pilocarpine which is due to the stimulation of ganglion cells would also be removed by cooling.

Potentiations by eserine; use of choline and of 2268F. If we think of a drug action as having possible muscular and nervous components, then it should be possible to distinguish the presence of the latter with the help of eserine. Thus it had been suggested that any drug which is potentiated by eserine must owe at least part of its action to a nervous or cholinergic component. Conversely, any drug which is endowed with only a muscular component should fail to be potentiated by eserine (unless it is hydrolysable by cholinesterase). Emmelin & Feldberg (1947) tested the second half of this proposition, but they chose for their 'controls' two drugs, choline and 2268F, both of which possess a nervous (ganglionic) component. Both drugs were potentiated by eserine, which led Emmelin & Feldberg to conclude that the criterion of eserine-potentiation was non-specific. Since they departed from a false premise, this, their conclusion, would appear to be invalidated.

There is one difficulty in applying this criterion, namely that eserine, administered by itself, slowly causes the gut to contract and then throws it into rhythmic contractions. It is evident then that any apparent potentiation of another drug by eserine might be due to the summation of this slow rise in base line produced by eserine, together with the motor effect of the drug proper. However, the effect of a small dose of eserine can be graded, and it usually begins after a latent period of 30–60 sec. or more (see, for example, Ambache, 1946, fig. 16 *C* 3), and rises slowly at first. In all the other experiments of that paper the precaution had been taken of injecting the 'test'

drug whose potentiation was being studied, 5–10 sec. after the eserine, i.e. well before the beginning of any eserine contraction. Moreover, it is the fact that the immediate response to the 'test' drug shows potentiation, which is significant. This response occurs between the 10th and 30th sec. (usually maximal by the 15th–20th sec.) after the first injection of eserine, i.e. during the latent period.

Action of acetylcholine after nicotine; 'controls' with 2268F *and pilocarpine.* In considering the action of acetylcholine itself, I had suggested that, in view of its well-known nicotinic action, part of the response of the gut to this drug might be due to an indirect effect upon the ganglion cells, and that this part was removable both by nicotine paralysis of the ganglion cells, and by cooling. Emmelin & Feldberg 'controlled' this experiment with 2268F and pilocarpine. Their results are shown in fig. 1 of their paper. In this, equivalent doses of 2268F and acetylcholine were administered in alternation throughout the experiment. After the onset of nicotine-paralysis of the ganglion cells, the response to acetylcholine is reduced at first, but recovers later, *although the nicotine is left in the bath.** The initial reduction, which had been reported before, had been attributed (Ambache, 1946) to a removal of a 'nicotinic' component in the action of acetylcholine on the gut. Such an action is denied by Emmelin & Feldberg on the grounds that the action of 2268F is also reduced by nicotine, as shown in their figure. It is quite probable, however, that this reduction by nicotine of the response to 2268F, and to pilocarpine, is itself due to the removal of the ganglionic component of these two drugs.

The more recent experiments of Collins (1948) show that the action of acetylcholine on the gut is reduced (in ten out of thirteen experiments) by another ganglion-cell poison, namely tetraethyl ammonium bromide.

Experiments on acetylcholine production. Emmelin & Feldberg also studied the effect of KCl, $BaCl_2$, and histamine upon the acetylcholine production in the gut. They claim that these substances fail to increase the amount of acetylcholine released within the gut. If this were true, it would indeed be surprising, since it is well established that KCl releases acetylcholine from a wide variety of cholinergic nerve endings, and $BaCl_2$ has, as we have seen, an action upon ganglion cells, and also upon nerve fibres and endings (Feng, 1937; Dun & Feng, 1940), all of which would be expected to result in an increased release of acetylcholine. However, if we analyse Emmelin & Feldberg's results (p. 498) we see that their statement that 'potassium was ineffective or accelerated synthesis in one out of five experiments only' is inaccurate. The amounts of acetylcholine produced in their experiments have been calculated from their table and are set out in Table 1 below.

* This recovery from nicotine is not unlike that observed by Thomas & Kuntz (1926), who found that nicotine impaired the effect of vagal stimulation on the gut only at first, and that later, when they had also raised the dose of nicotine (25–50 mg. of nicotine/kg.), vagal function and therefore transmission, recovered: with still higher doses of nicotine the vagal effect was actually potentiated. It would seem that the action of nicotine on the ganglion cells in the gut presents certain anomalies which require further elucidation.

It will be seen that of the total of seven intestinal strips incubated with KCl, four (marked by asterisks) show a distinct increase in acetylcholine production over and above the control strips of the same experiment. Moreover, in this section of their paper, Emmelin & Feldberg did not repeat my original experiments under identical conditions. The main differences in our methods were: (*a*) in the concentration of eserine used, and (*b*) that, whereas I had used pieces of intestine 13–15 cm. in length, in Emmelin & Feldberg's experiments the intestines were cut into small pieces of 1 cm. It is possible that the very much greater injury produced by their procedure, acting itself as a stimulus, was responsible for some of the rather high values, e.g. 3 and 5·2 μg./g. in their control groups (col. 2).

In their experiments on acetylcholine synthesis in the presence of barium, again the conditions of my experiments were not closely observed. The total dose of $BaCl_2.2H_2O$ in my experiments was 2–4 mg. which represents a concentration of 1·7–3·4 mg. of $BaCl_2$ in 6·3 c.c. (i.e. 0·27–0·54 mg./c.c.), whereas Emmelin & Feldberg used a dose about 7–28 *times as large*, i.e. 4 and 8 mg./c.c. of $BaCl_2$. Now, we have seen from the present experiments that a large dose of barium may exert a depressing action upon the ganglion cells: this may account for some of Emmelin & Feldberg's low values. Of the strips incubated with $BaCl_2$, one had a negative value for acetylcholine production (− 2, in

TABLE 1. Production of acetylcholine (μg./g./40 min.) in intestinal strips (compiled from Emmelin & Feldberg, 1947, p. 498)

	Controls		KCl		$BaCl_2$	
Exp.	(1)	(2)	(3)	(4)	(5)	(6)
1	2·5	3·0	4·3*	—	2·6	—
2	2·1	2·7	2·8	—	2·7	—
3	1·8	1·7	5·0*	—	−2†	5·4*
4	1·4	5·2	2·5	3·6	—	—
5 (buffered)	0·9	2·1	3·5*	3·0*	4·0*	3·9*
Average	2·34		3·53		3·72 (omitting†) 2·77 (including†)	

Exp. 3) which is difficult to understand. Apart from this probably erratic result, three (asterisks) of the other five strips show a distinct increase in acetylcholine production over and above the control strips of the same experiment. In their last experiment (no. 5) the experimental conditions appear to have been improved by the introduction of a buffer. This experiment shows a clear-cut increase in acetylcholine synthesis produced by *both* KCl and $BaCl_2$.

The conditions of these experiments with KCl and $BaCl_2$ appear to have been so diverse that a statistical analysis on these few results is inconclusive, but 50 % of them appear to show a distinct trend. I am indebted to Mr D. A. Scholl, of the Department of Anatomy, and to Mr D. R. Westgarth, of the Department of Statistics, University College, for an analysis of variance (allowing for the disparity in the number of observations in the two groups) which demonstrated that a significance level of 11 % was associated with the differences between the KCl and control groups of results. This level admittedly does not indicate a marked difference between the two groups on the basis of this data, but is too unusual to dismiss, and it would be unwise to conclude that the observed difference was due merely to chance.

Is there such a thing as a purely smooth-muscle stimulating substance? It seems therefore, that, at the moment, it is difficult to specify a drug of which it can be said with absolute certainty that it is a purely muscle-stimulating substance devoid of side effects upon the nervous apparatus in the gut. We have seen why choline, acetylcholine, 2268F, pilocarpine, KCl and $BaCl_2$ will not serve the purpose. Muscarine itself deserves investigation, but it is known to have a nicotine-like action on the suprarenal medulla and excitatory effects upon the central nervous system.

As to histamine, it has a well-known action upon certain types of nerve endings. Thus it is capable of exciting axon reflexes in the skin, and the possibility of axon reflexes occurring in smooth muscle has been raised by Fischer 1944; their presence has been observed in the frog's lung by Dikjstra & Noyons (1939), and in the rabbit's lung (pleuro-pulmonary reflexes) by Reinhardt (1933). In the axon reflex of the 'flare' it is believed that A.Ch. is released by the nerve ending in contact with the blood vessels, since this ending, when excited by antidromic stimulation of the posterior roots, is capable of releasing A.Ch. (Wybauw, 1938). This may therefore be considered as a model of a system in which histamine starts off nerve impulses which eventually release A.Ch. Emmelin & Feldberg have found that benadryl suppresses the action of histamine on the gut without interfering with that of A.Ch. Now it has been shown (Parrot & Lefebvre, 1943) that histamine antagonists may prevent only the *initiation* by histamine of axon reflexes, but that they do not interfere with the rest of the reflex mechanism, since it can still be elicited by electrical stimulation of the axons in the skin, or of the posterior roots antidromically. Emmelin & Feldberg's findings with benadryl may be regarded as analogous to these, and are no proof that histamine cannot release A.Ch. in the gut, as in the skin.

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THE CIRCULATION TIME IN THE CAT, STUDIED BY A CONDUCTIVITY METHOD

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The time course of the action of certain drugs in the cat, particularly histamine liberators (MacIntosh & Paton, 1947), suggested that its relation to the circulation time should be investigated. We could find in the literature, however, no measurements of circulation time in the cat, and these experiments were designed to remedy this deficiency. The method used was to record the changes in the electrical conductivity of the blood after injection of substances of varying conductivity into the circulation. Stewart (1894), using rabbits and dogs, also used this method to measure the time between an injection and the first change in conductivity in the blood at various points. Others (see Ruskin & Decherd, 1947, for references) have recorded circulation times by the injection of substances known to act at various sites (e.g. decholin, saccharin, histamine, sodium cyanide, nikethamide). But all these methods have the disadvantage that they measure only one particular time; in general, that of the shortest path from the site of injection to some specific organ. It is clear that there may be a wide range of paths by which blood may travel between two points in the body, and we have tried by continuous recording to demonstrate the resulting range of circulation times, both in the greater and lesser circulation. In this paper we describe the method of recording time-concentration curves of a substance injected into the circulation and of deducing from these curves the average circulation time and the cause of its variation. It is hoped to discuss in a later paper the relation of such curves to the action of certain drugs (cf. Gray & Paton, 1948).

METHODS

Preparations. Cats under chloralose anaesthesia have been used in all experiments; they were eviscerated when necessary. After all dissection had been completed, time was allowed for capillary oozing to cease, and for firm clots to be formed. Heparin (2 ml. of 1% sodium salt) was then injected intravenously, and not less than 15 min. later, cannulae were inserted. Electrode cannulae, which were tied in continuity with a vessel, were inserted as quickly as possible to minimize disturbance of the circulation. The cannulae for injection were either glass cannulae tied

into a vein or were blunt needle cannulae which were inserted through small side vessels so that their tips lay in the vessel into which it was desired to inject. In the latter case adjacent branches of the vessel concerned were tied and divided.

Injecting system. The injecting system consisted of a graduated pipette fitted to a two-way tap. One arm of the tap was connected with a metal adaptor which could be plugged into the needle cannulae, and the other with a reservoir. In order to minimize distensibility, the junctions were kept as short as possible and made with plastic tubing. The other end of the pipette was connected with a pressure reservoir which enabled any chosen rate of flow to be maintained uniformly. In some experiments injections were made from a pipette into a glass cannula tied into the main vein.

Electrode cannulae. It was necessary to measure the resistance of the blood without interference from the resistance of the vessel wall or of the external fluid, or from changes in the length and diameter of the vessel. For this reason it was decided to mount the electrodes in a cannula which could be tied in continuity with the vessel. Three electrodes were used, the outer two being con-

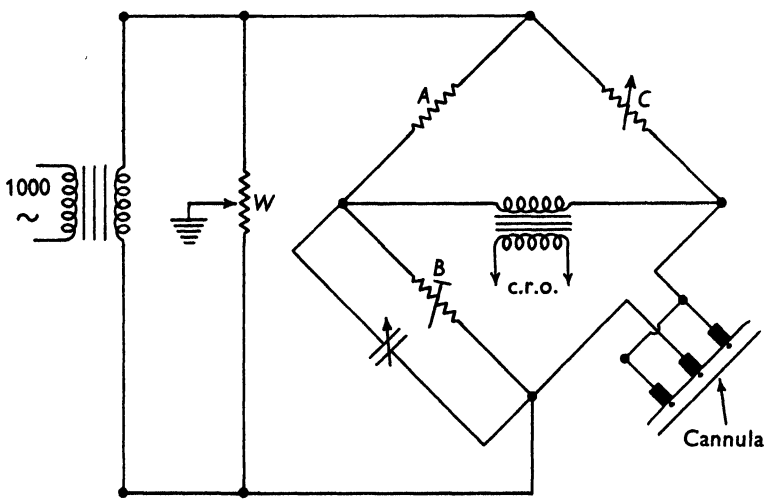

Fig. 1. Diagram of a.c. impedance bridge and cannula. Normal working conditions; $A = B = 2000\ \Omega$. Final balance of resistance obtained with C and the bridge balanced against earth with the potentiometer W.

nected, thus short circuiting the parallel resistance provided by the blood and tissues in contact with the ends of the cannula. As shown in Fig. 1, a glass tube of 3 mm. internal diameter was provided with three side arms 10 mm. apart into which 0·5 mm. diameter silver wires were fixed with de Khotinsky's cement. When filled with blood the cannula had a resistance of about 2000 Ω.; the true capacity of blood at low frequencies has been shown by Fricke & Morse (1926) to be of the order of 100 μμF. for a cubic centimetre of blood, the same volume having a resistance of the order of 200 Ω. This capacity would imply a reactance at 1000 cyc./sec. of about 10 MΩ. in parallel with our resistance and can clearly be ignored. Various minor modifications in the cannula were made at different times.

Bridge and recording system. The recording cannula formed one arm of a resistance bridge (Fig. 1). Resistances A and B were fixed equal to each other, usually at 2000 Ω. The bridge was balanced with resistance C, and the variable condenser was used to balance the capacity in the cannula. 3 V. r.m.s. 1000 cyc./sec. was supplied to the bridge through a transformer, and the balance was recorded through a transformer coupling with amplifier and cathode-ray oscillograph. A double-beam oscillograph was used without time-base deflexion and the spots were photographed on

moving paper. One beam was connected to the output of the bridge and the other to an oscillator to give a time scale. A second tube was fitted with time-base deflexion and was used for balancing the bridge. The only deliberate connexion from the bridge and animal to earth was through the Wagner earth (potentiometer W). This was adjusted so that, at balance, the output of the bridge was also balanced against earth.

Calculations

The amplifier and bridge were calibrated at frequent intervals by replacing the cannula with a decade resistance box. The calibrations were plotted so that a change of width from the null point represented the percentage change of resistance in the cannula. The calibrations were sufficiently linear near the null point for the change of width to be used over the range of the initial widths employed. Changes of capacity in the cannula were not detectable with the type of deflexions discussed in this paper.

TABLE 1. Resistance of mixtures of (A) blood and 0·9% NaCl, and of (B) blood and 5% NaCl

Mixture	Observed resistance (Ω.)	Expected resistance (Ω.)	Error (%)
	A		
Whole blood	2440	—	—
0·9% NaCl	1390	—	—
98% blood	2405	2405	Nil
95% blood	2350	2355	−0·2
90% blood	2270	2265	+0·2
80% blood	2145	2120	+1·2
50% blood	1775	1770	+0·2
	B		
Whole blood	1015	—	—
5% NaCl	113	—	—
99% blood	963	940	+2·4
98% blood	890	876	+1·6
90% blood	597	565	+5·7

If it be assumed that the final resistance in the cannula is the resultant of the parallel resistances due to the blood and the injected substance, then the final resistance (R) can be calculated as

$$\frac{100-F}{r_1}+\frac{F}{r_2}=\frac{100}{R}, \tag{1}$$

where r_1 = resistance of the cannula when filled with blood at the temperature of the mixture; r_2 = resistance of the cannula when filled with injected substance at the temperature of the mixture; and F = the percentage concentration of the injected substance in the cannula blood at the specified time.

Stewart (1894) and Fricke (1933) have shown that this relation is not accurate if r_1 and r_2 are taken to represent the resistivities of cells and plasma respectively. However, when whole blood is diluted, as much as 50% with 0·9% NaCl or 10% with 5% NaCl, the simple assumption holds within the accuracy of our method (Table 1).

In practice r_1 is measured from the balance of the bridge immediately before the injection; r_2 is measured in the same cannula immediately after the experiment, the necessary temperature correction being applied. The percentage change of resistance (x) is obtained from the record and equals $\frac{100\,(r_1-R)}{r_1}$. Substituting for R in equation (1) and transposing, we obtain

$$F=\frac{100xr_2}{(100-x)\,(r_1-r_2)}. \tag{2}$$

If the volume flowing through the cannula in unit time is h, then the total volume, (V), of the injected solution which has passed through the cannula is given by the equation

$$v = h\int F dt = \frac{hr_2}{r_1 - r_2}\int \frac{100x}{100 - x}\, dt. \tag{3}$$

The full numerical evaluation of this integral is tedious, but approximation is possible. In a typical conductivity trace, x may not exceed 5–7%, and the mean value is considerably less. It is then possible to replace $\frac{100x}{100-x}$ by x in equation (3), with an error less than 5%. $\int x dt$ represents the area of the tracing and can be conveniently measured with a planimeter. If the rate of blood flow is known, the volume of the injected solution passing through the cannula can thus be calculated. Conversely, if the volume injected is known, the rate of blood flow can be calculated when recording is from the site of injection.

For the case where a steady state is obtained, using a constant rate of injection (I) over a time t, we have

$$v = It = hFt. \tag{4}$$

This equation is used in the calculations of blood flow, described at the end of the next section.

It is interesting to consider what happens at a junction or division of the blood stream. At a junction, the concentration F in one branch at any time t is diluted by the blood from another branch in which $F = O$, giving $\frac{kF}{k+K}$ as the concentration in the mixed stream, where k is the volume in unit time flowing in the former and K that in the latter. The area of the time—concentration relation therefore equals $\int \frac{kF}{k+K} dt$, and the volume of injected substance is $(k+K)\int \frac{kF}{k+K} dt$, which equals $k\int F\, dt$, the volume of injected substance in the branch before mixing.

At a division the concentration F at a given time t must be the same at the mouths of both branches. Therefore $\int F dt$ will be the same in both mouths. Under constant conditions of blood flow this integral must remain constant as it passes down each branch. The volume of injected substance in each branch will be proportional to the two rates of flow, being equal to $c\int F dt$ and $C\int F dt$, where c and C are the volumes in unit time flowing in the two vessels.

It should be noted that the area $\int F dt$ is not altered by being measured in a branch (although its shape may change); for although the amount of injected substance flowing down one branch is $\frac{c}{C+c}$ times that passing down the main trunk, the rate of flow past the recording cannula is also $\frac{c}{C+c}$ times the rate of flow in the main trunk.

These considerations can be conveniently illustrated by a typical example. (In what follows it is assumed that the injected substance is not distributed in the blood by the process of flowing; even if this occurs, however, the main argument is still valid.) Suppose that after an injection into a vein there is at time t a concentration F' of the injected substance in the blood of the vein. If the rate of flow in the vein is k then the volume injected (V) will equal $k\int F' dt$. During its passage to the heart this blood will be mixed with uncontaminated blood from other veins giving a concentration of $\frac{kF'}{k+K}$ as it enters the heart; $(k+K)$ being the total volume of blood entering the heart. It is this concentration which leaves the heart and passes into the arteries, so that the value of F recorded in the carotid is equal to $\frac{kF'}{k+K}$. If c equals the volume of blood flowing through the carotid

in unit time then the volume of injected substance in the carotid will equal $c\int\frac{kF'}{k+K}dt$. This volume could, if desired, be calculated from the conductivity record, if c was known. Usually, however, the total volume (V) of injected substance is of more interest. This volume (V) must all pass through the heart, and since the concentration at the aortic valves is $\frac{kF'}{K+k}$ and the cardiac output is $k+K$, then $V=(k+K)\int\frac{kF'}{k+K}dt$. V is known and $\int\frac{kF'}{k+K}dt$ can be obtained at any arterial branch: hence the cardiac output $(k+K)$ can be calculated (cf. White, 1947). This can be rewritten as

$$V=U\int Fdt, \tag{5}$$

where V is the total volume injected, U is the cardiac output, F the percentage concentration measured after it has all passed through the heart and t is the time. More generally this formula gives the relation between the volume of substance (V) injected at any point, the area $\left(\int Fdt\right)$ of the time—concentration relation at any other point (either in a main trunk or in a branch), and the rate of flow (U) of all that blood with which the injected material mixes between the point of injection and the point of recording.

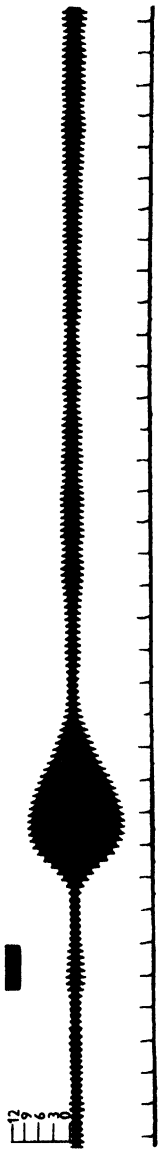

Fig. 2. Record of change in conductivity of carotid blood after the injection, during the signal, of 2 ml. of 0·9% saline into the femoral vein. Time, 1 sec. Ordinate = concentration % (F).

RESULTS

Control experiments

The injection into the circulation of a small volume of hypertonic sodium chloride solution, or of a larger volume of 0·9% sodium chloride causes the conductivity of the blood to increase. Fig. 2 is a record of the change of conductivity in the arterial blood after the intravenous injection of 2 ml. of 0·9% sodium chloride. (For this record and all others shown, the balance of the bridge was arranged so that an increase in the width of the tracing indicates an increase in conductivity of the blood in the recording cannula. Conversely, a decrease in width indicates a decrease in conductivity (cf. Fig. 3).) It was necessary to show that such a change of conductivity is due solely to the appearance between the electrodes of the injected substance and that this change accounts quantitatively for its total volume.

Causes of conductivity change. In the first place, the change in conductivity depends on the conductivity of the injected material. Thus, 0·9% NaCl which has a conductivity roughly twice that of blood causes an increase; on the other hand, an injection of isotonic dextrose, whose conductivity approaches zero,

causes a transient decrease in the conductivity of the blood. The sensitivity of the method, however, is such that changes in the conductivity of the blood due to other causes became significant; for example, small alterations in haematocrit value were easily detectable. The conductivity of the blood also depends on its rate of flow through the electrode cannula (this interesting phenomenon will not be discussed further in this paper, except in so far as it concerns our results). Finally, since the conductivity of an NaCl solution changes by about 2% per degree C., serious errors could arise were temperature not controlled.

The following experiments were done to test how far these factors were sources of error. In the first place, whole blood withdrawn from the animal and then reinjected had no effect on the conductivity. Similarly, a suspension of cells in 0·9% NaCl adjusted to the same proportion of cells as in the animal's blood, was without effect. These results appeared to exclude any contribution to change in conductivity from the mechanical effects of the injection itself. This conclusion was supported by finding that injections of histamine or adrenaline mixed with blood in doses adequate to produce a large change in blood pressure were also without effect on the conductivity of the blood within our normal recording period. A small change in conductivity was, however, observed, starting not less than 40–50 sec. after the injection.

Experiments with equiconductive solutions. Injections of isotonic dextrose solution caused, as mentioned above, a decrease in the conductivity of the blood. This implied that, besides whole blood or a suspension of cells in 0·9% NaCl, there should exist a mixture of 0·9% NaCl and isotonic dextrose solution equiconductive with blood, which would give no change in conductivity when injected. It was found, however, that such mixtures, although they were equiconductive with the blood of the animal used, caused a small increase followed by a small decrease in conductivity. Further, even after the injection of mixtures of definitely lower conductivity than the blood, this small increase preceded the main change in blood conductivity. Fig. 3 shows a typical series of diphasic responses. Such experiments suggested that the earlier portion of the injected mixture after entering the vein picked up electrolytes from, and lost glucose to, the tissues, thus becoming more conductive than subsequent portions. The restitution of the tissue electrolytes would account for the subsequent depression of conductivity. We have calculated that the peak change of conductivity is approximately 5·8% after the injection of an equiconductive solution (Fig. 3*b*). When various volumes of a solution of lower conductivity than the blood were injected the size of the hump did not increase with the volume of mixture injected, although the succeeding trough became larger and longer.

Electrolyte exchange. The experiments just described suggested that the injection of any solution with a solute concentration gradient to the tissues

may lead to an exchange of solutes affecting the change in conductivity. Exchange with the blood cells, however, does not appear to be important when blood is mixed with 5% NaCl (Table 1), for the observed changes in resistance agreed closely with those calculated on the assumption that all the electrolyte remained in the plasma. After injections of hypertonic NaCl one would expect these electrolyte changes to delay and flatten both rising and falling phases of the change of conductivity against time. Thus, in one experiment the mean femoral vein to carotid artery peak times were; with 0·9% NaCl, 7 sec.; isotonic dextrose, 8 sec.; and 22·5% NaCl, 8·5 sec. But in other experiments the times did not vary significantly with different solutions. The falling phase was usually prolonged and tended to obscure the gap between the first and second circulations. The possibility of such exchanges represents an argument

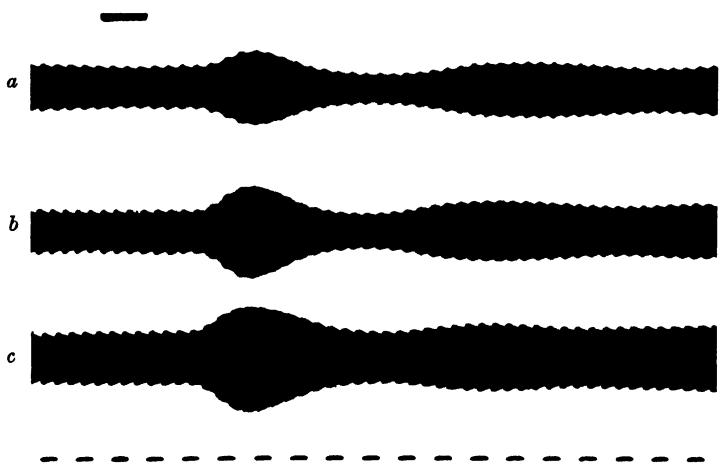

Fig. 3. Cat, 2·9 kg., chloralose. Injections into cannula tied into femoral vein of 5 ml. of various mixtures of 0·9% NaCl and isotonic dextrose; (*a*) 60, (*b*) 70, (*c*) 80% of 0·9% NaCl.

for the use of saline as an indicator, since it is isotonic and sufficiently similar in constitution to plasma not to lead to important solute exchanges, but it is clear that hypertonic NaCl can be used without serious error.

Velocity of flow. The sensitivity of the cannula to the velocity of the blood through it had two undesirable consequences; in an animal with low blood pressure there was a substantial variation in conductivity with each heart beat and sometimes even respiratory variation. The pulsation was only a technical nuisance, but the respiratory variation represented a change of a type and size sometimes to be confused with small changes due to injected saline. It was necessary, therefore, in some experiments to time the respiration and to relate it to the conductivity record, or to place the animal on artificial respiration. Unless the blood pressure was low, however, the respiratory and pulse variations were trivial and led to no confusion.

Temperature. Temperature errors can be considerable if an injection is made close to a recording cannula and the temperature is not controlled. When, however, the cannula is at some distance from the point of injection (for example, when it is in an artery and injections are made intravenously), it was found that the effect of the injected solution depended very little on its temperature. Injections of saline at body temperature or 20° C. below it produced changes in conductivity differing by less than 8%. It follows, therefore, that the injected fluid is rapidly warmed or cooled to body temperature. This heat exchange, however, cannot take place with the blood with which the fluid mixes, since this would alter its conductivity in proportion to the temperature of the injected solution. It must occur with some other heat reservoir, possibly the lungs in this instance.

Quantitative controls. The experiments described define the conditions in which it is possible to measure the conductivity changes due to the presence in the cannula of the injected substance. There is also evidence that the injected substance can be accounted for quantitatively. In the intact animal, it has been possible to show that the areas of the curves relating conductivity change to time (blood flow being constant) are proportional to the volumes of the injected substance (Table 2). Using an *in vitro* model it has been possible to go further and, by measuring the blood flow, to calculate within 10% the volume of substance which has, in fact, been injected (Table 3).

Finally, experiments were done on the reproducibility of the changes in conductivity of arterial blood after the injection of 0·9% NaCl intravenously. Table 4 gives the results of one such experiment.

The values in Table 2 of the ratio of the area of the conductivity tracing to the volume of saline injected are of interest. This ratio for the auricle-to-carotid route is larger than for the vein-to-carotid route, and this could be accounted for by a fall in cardiac output caused by opening the chest. A similar comparison between the vein-to-carotid route and the femoral artery to femoral vein route gives an estimate of the femoral blood flow as one-tenth of the cardiac output (this is based on the assumption that the blood passing down the femoral artery is not later mixed with any significant volume of blood free of saline from another arterial source).

Properties of the circulation

Circulation time after rapid injection. Fig. 2 shows the record obtained from the carotid cannula after injecting 2 ml. of 0·9% saline into the femoral vein. In this case the conventional technique was used, a glass cannula being tied into the femoral vein and the saline injected rapidly from a syringe or a pipette. Here the injection time was 1·1 sec. as can be seen from the signal. Sharp spikes on the record indicate the heart beat and the respiration can just be seen as a shallow undulation occurring at approximately 2 sec. intervals. The

frequency and amplitude of the respiratory change are such that they cannot be confused with the slower and larger waves due to the circulation of the injected substance. The resistivity of the blood was measured immediately before the

TABLE 2. Relation of the area of the conductivity tracing to the volume of saline injected

	Injection time (sec.)	Injection vol. (ml.)	Area (mm.^{2})	Ratio (area/vol.)
Auricle to carotid artery	2	0·267	39·8	149
	5	0·576	82·8	144
	10	1·158	152·4	132
Femoral vein to carotid artery	2	0·182	20·5	113
	5	0·391	41·9	107
	10	0·829	86·9	105
Femoral artery to femoral vein	2	0·070	74·9	1070
	5	0·191	230·3	1220
	10	0·360	352·3	980

TABLE 3. Measurement of blood flow in an *in vitro* model

Rate of injection (ml./sec.)	Rate of blood flow (ml./sec.) Observed	Calculated	Deviation (%)
0·297	1·43	1·28	−10·5
0·268	1·47	1·47	0
0·267	1·43	1·51	+5·6
0·235	1·30	1·27	−2·3
0·225	1·40	1·45	+3·6
0·202	1·47	1·47	0
0·201	1·40	1·48	+5·7
0·190	1·43	1·37	−4·2
0·148	1·43	1·36	−4·9
0·145	1·37	1·35	−1·5
0·142	1·47	1·66	+12·9
1·126	1·40	1·48	+5·7
0·0938	1·40	1·35	−3·3
0·0937	1·50	1·54	+2·7
0·0931	1·43	1·56	+9·1
0·0688	1·37	1·34	−2·2

Mean deviation = 1·03 %
Root mean square deviation = 5·81 %

TABLE 4. Reproducibility of changes of conductivity after injections of 0·9 % NaCl. Six successive injections of 2 ml. into femoral vein. Recording from carotid artery

Time to start (sec.)	Time to peak (sec.)	Peak amplitude (mm.)
3½	5½	24
3	5½	23½
3½	5½	22½
3	5	20
3	5	18½
3½	5½	21½

record was taken, and that of the 0·9 % saline at the end of the experiment. The resistivity of the saline was corrected to the rectal temperature of the animal at the time of the injection. From these figures, and the calibration of the instrument, the scale of percentage concentration of injected substance (F) was constructed (see p. 175).

The first passage of the saline past the electrode is clear; it starts $3\frac{1}{4}$ sec. after the beginning of the injection, reaches a peak $F = 8{\cdot}8$ at $5\frac{1}{4}$ sec., and returns to its original base line at 9 sec. At 15 sec. another peak with $F = 1{\cdot}1$ occurs which, because of its shape, timing and regularity of occurrence is believed to be a second circulation of the saline through the cannula. The base line does not again return to its original level during this record, but there is a slight depression at $20\frac{1}{2}$ sec. before it takes up a new steady level at $F = 1$. From this level it returns after 5–10 min. to the original base line. The value of $F = 1$ for the uniform mixture of 2 ml. of saline with the blood implies a total circulating blood volume of 200 ml., which agrees well with the expected blood volume.

TABLE 5. Circulation times in the cat

	No. of cats	Time from beginning of injection to peak of response (sec.) Mean	S.E. of mean
Femoral vein to carotid or aorta	13	6·3	0·32
Femoral vein to carotid or aorta (second time round)	8	18·2	1·43
Aorta to inferior vena cava	3	12·5	1·03
Aorta to carotid or aorta	8	14·1	1·30

(Aorta; injection cannula tied into inferior mesenteric artery.)

TABLE 6. Effect of volume and speed of injection on circulation time (femoral vein to carotid artery)

Exp.	Vol.	Injection time (sec.)	Injection velocity (ml./sec.)	Start to start (sec.)	Mean to peak (sec.)
31. x. 47	1	0·4	2·5	$5\frac{1}{2}$	$7\frac{1}{4}$
	2	0·6	3·3	$4\frac{1}{2}$	$6\frac{3}{4}$
	5	1·0	5·0	4	$6\frac{1}{2}$
	5	1·1	4·5	$3\frac{1}{2}$	$5\frac{1}{2}$
	10	1·9	5·3	$3\frac{1}{2}$	$5\frac{1}{2}$
19. xi. 47	3	15·7	0·2	$5\frac{1}{2}$	7
	3	7·3	0·4	4	$6\frac{1}{2}$
	3	4·2	0·7	$3\frac{1}{2}$	$5\frac{1}{2}$
	3	2·5	1·2	$3\frac{1}{2}$	$4\frac{3}{4}$
	3	1·1	2·7	$2\frac{1}{2}$	4
	3	1·0	3·0	$2\frac{1}{2}$	$4\frac{1}{2}$
	3	0·5	6·0	$2\frac{1}{2}$	$3\frac{3}{4}$

Advantages of this method. Table 5 summarizes circulation times obtained by this method. This type of record gives a direct picture of the distribution of an injected substance in the arterial blood after a common type of injection. 0·9% NaCl is the substance injected, so that diffusion of electrolytes is unlikely to have distorted the record. The record is calibrated as percentage concentration of injected substance; hence, if a drug is mixed with the saline and it is not absorbed specifically, then the distribution of the drug in the arterial blood is known. Information about starting and peak concentration times can be obtained, and the brevity of the injections allows the second responses to be seen. Nothing can, however, be said about the physiological scatter that occurs, since much of the spread seen here could occur in the process of injection.

Velocity and volume of injection. Even starting and peak concentration times are distorted when using this technique, since they depend on the velocity of injection. If, for example, a cannula is tied into the femoral vein the velocity of the saline cannot depend on the blood velocity until it reaches a major venous junction, such as that of the external and internal iliacs. For the early part of its course its velocity must depend entirely on the velocity of injection. The results given in Table 6 show that for the same volume injected reduction of the velocity of injection increases the measured circulation time.

These results also show that for a given velocity of injection a change in volume has little effect on the time recorded. This suggests that during injections such as these, in which the pressure is high compared with that of the venous

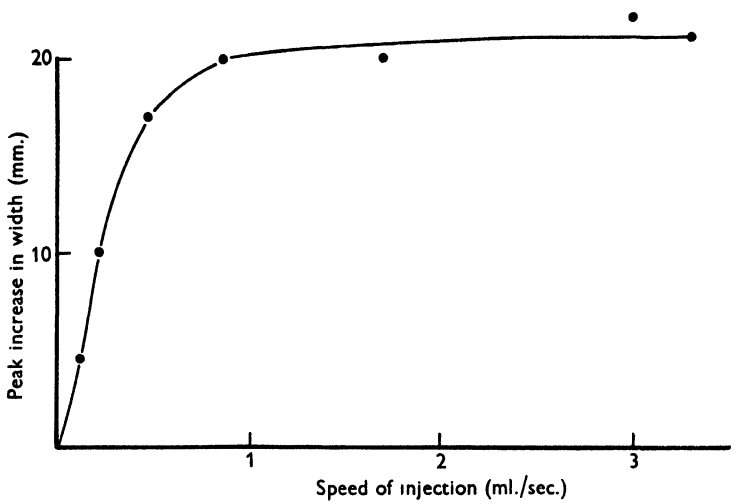

Fig. 4. Relation between peak increase in width of conductivity tracing and speed of injection of 0·9% NaCl into cannula tied into femoral vein. Cat, 3·4 kg., chloralose. Recording cannula in aorta.

blood, the injected solution spreads out into the venous bed almost equally with and against the stream; if the veins are exposed this process can indeed be seen. This implies that the errors of the method will be least if injections are made at a pressure high compared with the venous pressure and are of a volume which is small compared with that of the vascular bed between the sites of injection and of recording; the measured circulation time is then that from the first major confluence. Also, if an injection is sufficiently rapid to displace the blood entirely, a length of vessel at the main confluence will be filled with injected solution, and further increase in speed of injection will not increase the proportion of it there. Its peak concentration in the arterial blood will therefore approach a limiting value as the velocity of injection is increased, and will be independent of it over a wide range. This can be seen in Fig. 4 in which peak

concentration of saline in the aorta (expressed as increase in width of the record) is plotted against injection speed.

Measurement of the dispersion of the injected substance in the circulating blood

Preliminary observations. The experiments so far described have been concerned particularly with the size and time of the maximum change of conductivity of the blood after an injection into the circulation. These two measurements alone do not completely describe a record such as Fig. 2. For instance, in this record the conductivity begins to change 2 sec. before and does not return to its original level until $3\frac{3}{4}$ sec. after the peak, although the injection only lasted $1\frac{1}{2}$ sec. It follows that elements of injected material which entered the circulation at the same instant must have arrived at the recording cannula at widely different times. This is tantamount to saying that there are many different 'circulation times' between any two points of the circulation; and consequently a measure of this variation must be included if a complete description of the 'circulation time' is to be given.

The experiment illustrated in Fig. 5 was an early attempt to demonstrate this scatter by a direct comparison of records obtained as close as possible to the point of injection with those obtained at more distant points. Recording cannulae were tied into the inferior vena cava and aorta, and injections were made into the lumen of one or other of these vessels about 1 cm. upstream of the recording cannula. Fig. 5*a* shows the initial scatter after an injection into the inferior vena cava; this can be regarded as an approximately rectangular wave of injected material in the blood, of the same duration as the injection. Fig. 5*b* shows the wave after it has passed through the heart and lungs; Fig. 5*c* (latter part) the result of a complete circulation back to the inferior vena cava; and Fig. 5*b* (latter part) the result after $1\frac{1}{2}$ circulations back to the aorta again. Fig. 5 (*d–f*) shows a similar series starting from the aorta. These curves demonstrate clearly how the wave lengthens as it passes round the body; but since the circulation times were unusually long owing to the condition of the animal, no stress should be laid on the quantitative aspect.

These records can be regarded as frequency distribution curves of a population leaving the site of injection during the period of the injection. Ideally, it is the frequency distribution of the population leaving a single point in the circulation at a single instant under undisturbed conditions, which should be described. Experiments like the one just quoted had the great disadvantage that the operative procedures involved depressed the blood pressure and slowed the circulation rate. For describing the circulation quantitatively, therefore, a simpler procedure was adopted.

Quantitative measurements. In the first place, the necessity for recording the shape of the conductivity wave close to the site of injection was removed by limiting the speed of injection to a maximum of 0·1 ml./sec. This speed is small

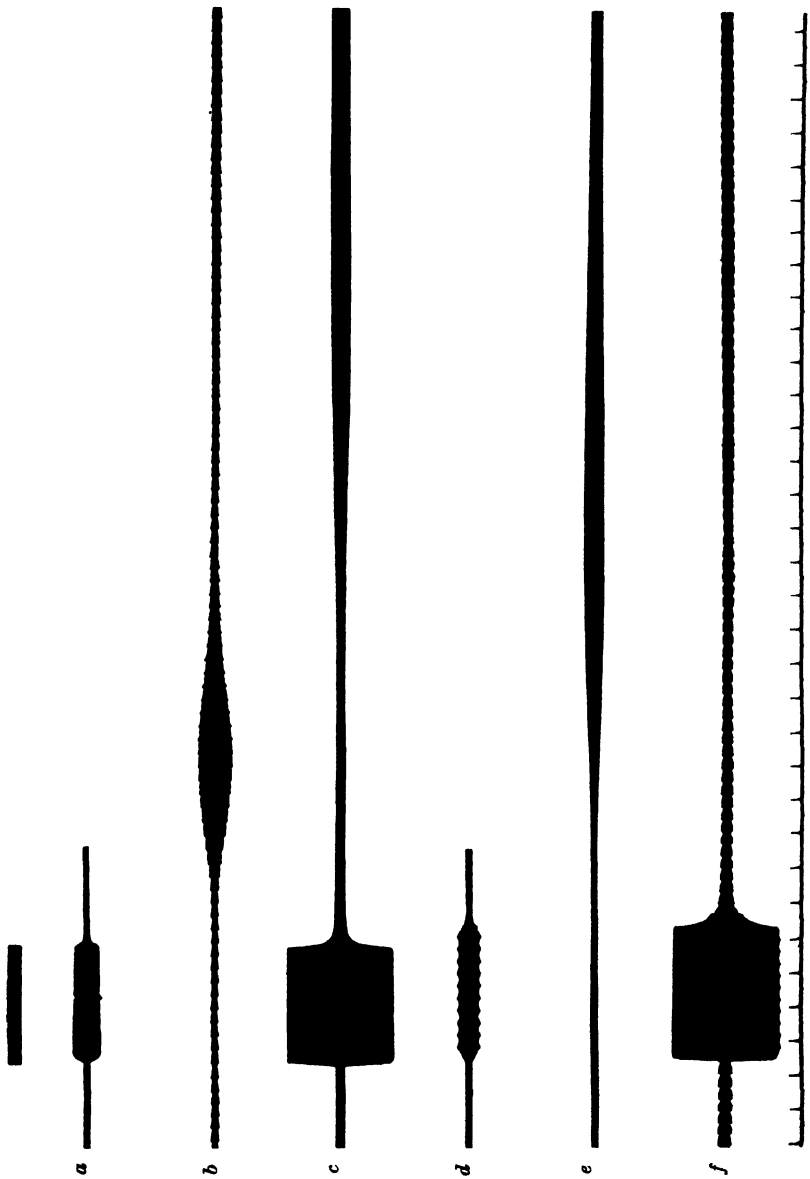

Fig. 5. Cat, 3·2 kg., chloralose. Recording cannulae in inferior vena cava and aorta. (*a*)–(*c*), injection of 3 ml. 0·9% NaCl into unobstructed inferior vena cava: (*a*) recording from vena cava (low amplification); (*b*) recording from aorta; (*c*) recording from vena cava (high amplification). (*d*)–(*f*), injection of 3 ml. of 0·9% NaCl into lumen of aorta: (*d*) recording from aorta (low amplification); (*e*) recording from vena cava; (*f*) recording from aorta (high amplication). Time, 1 sec.

compared with the velocity of blood flow in the blood vessels concerned, so that the resulting conductivity wave is rectangular, and its area proportional to the injected volume. This small injection velocity, however, meant that to obtain a measurable change in conductivity it was necessary to use injections of 3% NaCl lasting 2 sec. or longer; 2, 5 and 10 sec. injections were used, in order to allow extrapolation back to an instantaneous injection. The great advantage of this procedure was that it permitted the use of vessels accessible without major operative interference.

Three routes were investigated to show the effects of passage through the left heart alone, through both sides of the heart and pulmonary circulation, and through a limb. The details were as follows:

(*a*) A needle cannula was tied into the left auricle, and records were taken from the right carotid artery in the neck.

TABLE 7. Volumes and speeds of injection in experiments on the distribution of injected saline

	Theoretical injection time (sec.)	Injection time (sec.)	Vol. ml.	V/T
Left auricle to carotid	2	2·04	0·267	0·131
	5	5·10	0·576	0·113
	10	9·90	1·158	0·117
Femoral vein to carotid	2	2·14	0·182	0·085
	5	4·94	0·391	0·079
	10	10·01	0·829	0·083
Femoral artery to femoral vein	2	2·01	0·070	0·035
	5	5·17	0·191	0·037
	10	10·02	0·36	0·036

(*b*) A needle cannula was tied into a small branch of the right femoral vein just below the inguinal ligament, so that the tip of the cannula lay in the lumen of the femoral vein. Records were obtained from the right carotid artery.

(*c*) Injections were made into the lumen of the left femoral artery by tying a cannula into a small branch just below the inguinal ligament. The recording cannula was tied in the left femoral vein below the inguinal ligament.

The routine was carried out in five cats. In each experiment the records from the left femoral vein were taken first, followed by those from the femoral vein to carotid. The chest was not opened until these results had been obtained. In all experiments the blood pressure remained steady until the chest was opened.

Table 7 shows the mean times, volumes and rates of injection for each injection time and each route.

All records tended to have a wider, i.e. more conductive base line at the end than at the beginning. For vein to carotid and auricle to carotid records this was of the size to be expected from recirculation, but for the leg the changes were bigger than the recirculation of the small volumes used could account for, and may have been due to a slow trickle from capillary beds that were almost closed and had very long circulation times. However, before the data could be

handled it was essential that all curves should be brought to a finite end, even if a small distortion in the final result occurred. For this reason arbitrary base lines had to be adopted. With the auricle to carotid and vein to carotid records the base line was assumed to continue at its pre-injection level until recirculation began and then to rise linearly to the final base line. The point at which recirculation began was arbitrarily assumed for each cat as the time of the peak after a 2 sec. injection. This point was known to be approximately correct from other records. The limb records were corrected by assuming a linearly rising base line throughout the record.

All records were measured, the base lines subtracted, and the results plotted independently. The mean, mode and standard deviation of each individual curve was calculated. Table 8 gives the means and standard errors of these three statistics, of the original curves, together with the statistics of the

TABLE 8. Statistics of the circulation time

	Injection time (sec.)	Time from mean injection time to mean circulation time (sec.)		Time from mean of injection time to mode of circulation time (sec.)		Standard deviation of circulation times (sec.)	
		Mean of 5 exps.	S.E. of mean	Mean of 5 exps.	S.E. of mean	Mean of 5 exps.	S.E. of mean
Thoretical initial distribution	2	—	—	—	—	0·6	—
	5	—	—	—	—	1·4	—
	10	—	—	—	—	2·9	—
Left auricle to carotid artery	2	2·9	0·47	—	—	1·5	0·25
	5	3·0	0·33	—	—	2·1	0·11
	10	2·4	0·79	—	—	3·1	0·08
Femoral vein to carotid artery	2	10·1	0·59	10·0	0·64	2·9	0·29
	5	9·3	0·48	9·0	0·44	2·8	0·22
	10	8·4	0·44	9·5	0·29	3·5	0·23
Femoral artery to femoral vein	2	13·0	0·53	12·5	0·62	4·6	0·38
	5	12·2	0·44	11·1	0·83	4·7	0·43
	10	10·7	0·59	10·6	0·30	4·4	0·27

theoretical rectangular injection distributions. It will be seen that with the 2 sec. injection the standard deviation rapidly increases as the blood passes through the sections of the circulation. With the longer injection times this increase of standard deviation is obscured by the length of the injection time. The mean and modal times are shorter for long injections than for short. The fact that they do not agree within reasonable limits of error with the times given in Table 6, together with the relation of these results to the parameters of the frequency distribution of a population leaving a given point at one instant, will be discussed below.

The individual curves were all corrected to have equal areas and were alined on their means. The curves were then averaged over the five cats, giving nine mean curves illustrating three injection times through the three routes. These, together with the theoretical initial distribution, are shown in Fig. 6. In this figure the areas of the curves are proportional to the theoretical injection times,

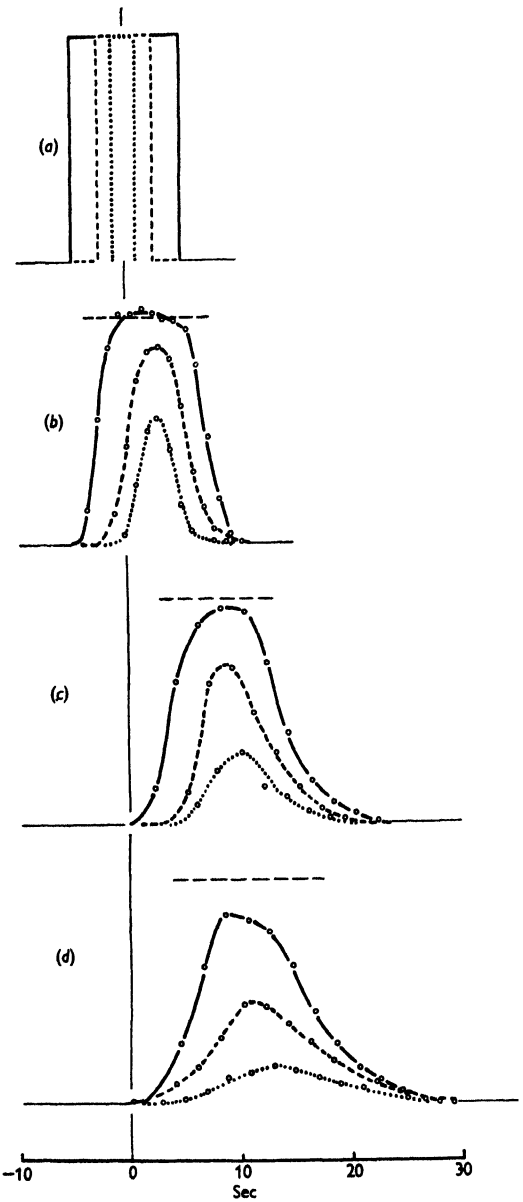

Fig. 6. Distributions of change of conductivity against time (mean of five cats); (*a*) Theoretical initial distribution; (*b*) injection into left auricle, recording from carotid artery; (*c*) injection into femoral vein, recording from carotid artery; (*d*) injection into femoral artery, recording from femoral vein. O———O, 10 sec. injection; O- - - - -O, 5 sec. injection; O.....O, 2 sec. injection. For other details, see text.

and zero time is taken as the mean of the injection. Doted lines indicating the height of the rectangles have been drawn above each curve.

DISCUSSION

The main object of our experiments has been to describe how a solution is distributed throughout the circulation immediately after it has been injected into a vein. The final quantitative experiments, using a technique adequate to provide such information, are summarized in Fig. 6 and Table 8. Before discussing the results, however, it is necessary to consider how far physiological conditions have been distorted by using hypertonic saline in these experiments, and what is the cause of the discrepancy between the circulation times then observed and those observed with rapid injections of saline into a cannula occluding the femoral vein.

We have already mentioned the technical reasons for which we were obliged to use a hypertonic solution; but we believe that this has not distorted the results significantly. It is indeed probable that some exchange of electrolyte always occurs between the tissues and an injected fluid, where these differ in electrolyte content; the experiments with 'equiconductive' solutions illustrate this exchange. But it is impossible to predict from these results in which electrolyte passes from tissues to injected fluid, and in which the volume of injected fluid is large, what would happen if small volumes of hypertonic saline were introduced into the circulation. We have already shown that passage of electrolyte into red cells is negligible, but we have not studied directly the possibility of exchange elsewhere. The results in Table 2 make it unlikely that this is important, since it is improbable that the exchange would always affect the same proportion of the injected material independently of its volume. Stewart (1921), using hypertonic saline, White (1947) using 3–5% NaCl, and Hamilton & Remington (1947), using thiocyanate, give evidence that disappearance of injected electrolyte from the blood during its passage from a vein to an artery does not exceed about 10%. Finally, our experiments with isotonic dextrose and with strongly hypertonic solutions gave circulation times close to those obtained with saline, and the shape of the conductivity tracing was altered but little. Cardiovascular reactions to hypertonic solutions have been described (e.g. Weed & McKibben, 1919), but this was with larger volumes of stronger solutions than we have used, and we have observed no changes whatever in blood pressure or pulse rate as a result of our injections.

The discrepancy between the two estimates of circulation time from femoral vein to carotid artery (Tables 6 and 8) is substantial and significant. But there are at least three factors tending to accelerate the circulation after a rapid injection, of 2–3 ml. volume, into a cannula tied into the femoral vein. The first is that the injection itself speeds the passage of the saline up the vein to the iliac bifurcation, instead of leaving it to be carried by the femoral blood flow.

This might shorten circulation time by about one second. Secondly, with volumes of this size it seems probable from Table 6 that while the injected saline spreads both up- and down-stream when it reaches the iliac bifurcation (as we have discussed), it, nevertheless, spreads somewhat more towards the heart than away from it; after correcting the figures in Table 6 for differences in velocity, we estimate that the reduction in circulation time from this cause is about $\frac{3}{4}$ sec. Finally, and possibly most important, the introduction of saline into the circulation alters the viscosity of the blood considerably, since blood is at least four times as viscous as aqueous solutions of electrolytes. Direct inspection of the inferior vena cava after rapid injections of 2–3 ml. of a solution into the femoral vein shows that the blood is much diluted. There must be, therefore, a lowered resistance to flow in the vena cava and through the heart and lungs, as the column of diluted blood passes. We have estimated that this reduction in blood viscosity could well shorten the circulation time by 1–2 sec., and possibly more. These corrections taken together can account completely for the discrepancy. There is, however, one point for which we have at present no satisfactory explanation. In our final experiment the interval from the middle of the injection period to the peak or mean of the response (Table 8) diminishes as the injection period is lengthened. This does not appear to be due to electrolyte exchange, viscosity changes or recirculation; but it is of interest that the diminution of this interval appears to be associated with some degree of 'skewness' of the distribution (Fig. 6).

The discussion of these details may serve to emphasize the extent to which estimates of circulation time may vary with conditions of measurement. To those we have mentioned (salinity, volume and viscosity of injected material, and speed and method of injection) must be added such factors as anaesthesia and body temperature. The times in Table 8 represent the speed of the undisturbed circulation in a cat anaesthetized with chloralose, but are of restricted application. Those in Table 6 are of more general application, being determined with rapid injections such as are commonly used of 2–3 ml. into an occluded femoral vein. Although such comparisons are difficult, it is of interest that the values recorded are intermediate between those recorded for the anaesthetized dog (Hamilton, 1928) and for the anaesthetized rabbit (Stewart, 1894).

Besides the interval between injection and appearance of injected material at the recording cannula, two other statistics are of importance. The first is the peak concentration. This varied with the speed of injection, and from cat to cat, but as a rule the peak percentage concentration of the injected substance in the carotid blood lay between 2 and 10%, after an injection into the femoral vein; after a further passage of the circulation, the peak concentration had fallen to not more than 1%. Thus, the injected material is almost completely mixed with the blood in one circulation.

The rapidity of mixing is relevant to the discussion of the other statistic, the

dispersion in time of the concentration of injected material round its mean time. The magnitude of this dispersion for an instantaneous injection can be estimated from Fig. 6 and Table 8 by extrapolation. It will be seen that whereas the variance of the distribution increases with increase in duration of injection for the rapid L-auricle to carotid artery route, it changes less for the femoral vein to carotid artery and still less for the femoral artery to femoral vein routes. By extrapolation the approximate values of 1·1, 2·5 and 4·5 sec. respectively are obtained for the standard deviations of the distribution that would follow an instantaneous injection.

Some of the dispersion might be due to turbulent mixing of the injected fluid with blood in front of and behind it; this mixing will be at its greatest in the left heart, but after passage through the heart an injection still appears as a nearly 'rectangular wave'. Alternatively, if the flow is stream-lined, scatter might be caused by 'coning' because the velocity of the centre of a fluid stream is greater than that at the sides. The maximum velocity of the fluid passing down a tube in laminar flow is twice the average velocity, regardless of the diameter of the tube: it is to be expected, therefore, that the dispersion of injected material from this cause would depend on the distance traversed, and not on the characteristics of the vessels through which the blood flowed. But this is obviously not the case: the mean distance along vascular paths in a 2·5 kg. cat from the femoral vein cannula to the carotid cannula is of the order of 40–50 cm., while that from the point of injection into the femoral artery to the recording cannula in the femoral vein cannot have been more than 25 cm. Yet Fig. 6 and Table 8 show a much greater dispersion in the latter than in the former circumstances. The dispersion introduced by the femoral capillary bed is the less to be expected in view of the observation that breaking up a tube of large cross-section into many smaller tubes leads to flow of less dispersion (Henderson, Chillingworth & Whitney, 1915; Stewart, 1898). Therefore, although mixing and coning may well explain the dispersion observed for the left auricle to carotid and femoral vein to carotid routes, it appears quite inadequate to explain the dispersion caused by the femoral artery to femoral vein circuit.

The remaining cause of dispersion to be considered is that there may be a wide variety of vascular paths, some fast and some slow, through which the blood passes between the main vascular trunks. This is probable on general grounds, since it is unlikely that an absolute constancy of length and diameter of arteries, capillaries and veins, is maintained throughout a tissue. It is at present impossible to calculate precisely the proportion of the dispersion due to this cause. It is, however, reasonable to suppose that the variance for the femoral artery to femoral vein time-distribution curves, due to causes other than a variety of vascular paths, cannot exceed the variance of the femoral vein to carotid artery curves, and is probably somewhat less. If we then assume that the variance of the femoral artery to femoral vein dispersion is made up of two

additive components, that due to a multiplicity of paths and that due to other causes, we can subtract from the total variance observed for this route that observed for the femoral vein to carotid artery route, so obtaining a minimum value of the dispersion due to multiplicity of paths. Thus the required variance is $(4{\cdot}5)^2-(2{\cdot}5)^2=14{\cdot}0$ sec. and $\pm 2\times\sqrt{14{\cdot}0}$ gives the range of time (14·8 sec.) within which the circulation through 95% of the paths is completed. The dispersion due to the capillary bed of the lungs must in any case be relatively small compared with that due to the capillary bed of the leg: for if it contributed at all significantly to the variance of the femoral vein to carotid passage, our estimate of the variance for the leg circulation must be correspondingly increased.

This degree of dispersion in the leg is substantial, but there is sufficient evidence that it is a possible outcome of normal vascular function. The existence of arteriovenous anastomoses is well recognized, and can account for rapid transit from artery to vein. But further than this, the metarterioles and precapillary sphincteric mechanism recently analysed by Chambers & Zweifach (1944) render possible a variability of vascular path of the type and extent required by our results. We suggest, therefore, that at least that part of a dispersion which cannot be accounted for by turbulent or laminar mixing, reflects a substantial variation of length and cross-section of circulatory paths existing in the intimate vascular structure of the tissues, and that such dispersion is considerable in the circulation of the leg and slight in the pulmonary circulation.

SUMMARY

1. Certain properties of the circulation have been investigated, in cats anaesthetized with chloralose, by continuously recording the changes of electrical conductivity of arterial and venous blood caused by the injection of solutions of various conductivities into the circulation. Our technique was sensitive to a change of conductivity of 0·5%.

2. Control experiments have shown that these changes are due to the injected material, and that this can be accounted for quantitatively, provided consideration is given to the temperature of the injected substance and to constancy of blood flow through the recording cannula. Errors due to electrolyte exchange after the injection of hypertonic solutions were estimated to be less than 10% of the conductivity change.

3. With these precautions, the conductivity change observed is related by a simple formula to the concentration of the substance in the blood. A further relation is derived which enables blood flow to be calculated.

4. A brief injection of a saline solution into the circulation causes a transient change in the conductivity of the blood, which is more or less prolonged according to the vascular path between the point of injection and the point of

recording. Records of this change show the distribution of the injected material in the blood. This distribution can be conveniently described by the mean and standard deviation of its time and by its peak concentration and area.

5. Circulation times in the greater and lesser circulations have been measured by recording the time interval between the beginning of a rapid injection of salt solution into one vessel and the moment of maximum conductivity change in another.

6. The distributions in the blood of injected salt solution have been analysed. The means, modes and standard deviations have been measured for the three routes: left auricle to carotid artery, femoral vein to carotid artery, and femoral artery to femoral vein. The characteristics of the circulation which cause these distributions are discussed.

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ON THE ACTIONS OF NORADRENALINE, ADRENALINE AND ISOPROPYL NORADRENALINE ON THE ARTERIAL BLOOD PRESSURE, HEART RATE AND MUSCLE BLOOD FLOW IN MAN

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A substance apparently identical with noradrenaline has recently been found in several animal tissues (von Euler, 1946); it has also been obtained in man, from an adrenal tumour (Holton, 1948), and from the blood of an adrenal tumor patient (Vogt, 1949). Noradrenaline may transmit certain sympathetic nerve impulses chemically (Bacq, 1933; Gaddum & Goodwin, 1947; Peart, 1949; Bulbring & Burn, 1949). It is therefore important to find out its actions on different parts of the body and to compare them with those of adrenaline, especially in man. Goldenberg, Pines, Baldwin, Greene & Roh (1948) found that noradrenaline slows the human pulse, diminishes the cardiac output and increases the peripheral resistance; its action in each case being the opposite to that of adrenaline.

Allen, Barcroft & Edholm (1946) investigated the action of adrenaline on the circulation in human skeletal muscle; the experiments described here were done with the object of finding out the effects of noradrenaline. The actions of noradrenaline and adrenaline on the heart rate and arterial blood pressure were also examined, as also the action of *N*-isopropylnoradrenaline ('isopropyl'). In the dog Konzett (1940 *a*, *b*) found that isopropyl dilates the bronchi and increases the heart rate and the cardiac output. In these respects it acts like adrenaline, only more powerfully; unlike adrenaline it does not usually cause vasoconstriction—it dilates the blood vessels in the gut and limbs (Konzett, 1940 *b*) as well as in the lungs (Hebb & Konzett, 1949) and in the skin (Marsh, Pelletier & Ross, 1947).

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METHODS

The experiments were done on normal subjects aged 25–45. Room temperature was rather low, usually between 16 and 20° C.

In the body a natural sympathomimetic substance is likely to act for minutes rather than seconds, so the solutions of the drugs were always given by an infusion lasting several minutes rather than by a rapid injection.

Intra-arterial infusions. In one series of experiments the drugs were given intra-arterially to avoid their action on the heart and to study their direct effect on the peripheral circulation.

The infusion apparatus consisted of a mechanically driven 50 c.c. syringe connected to a 100 cm. length of transparent 'telcothene' tubing about 1·5 mm. internal diameter into the end of which a piece of hypodermic needle about 4 cm. long and 0·8 mm. external diameter was firmly fixed with dental wax. (We are grateful to Prof. E. P. Sharpey-Schafer for showing us the advantage of the transparent tubing.) Before the experiment the apparatus was sterilized and filled with saline.

The techniques of intra-arterial infusion and measurement of the blood flow distally on the same limb were most easily combined on the leg as follows. The subject lay flat on his back so that the femoral artery was stretched. A plethysmograph was fitted to the calf and filled with water kept at 33–34° C. (Barcroft & Edholm, 1943). The arrangements for recording the calf blood flow were completed. The course of the femoral artery below Poupart's ligament was palpated and marked on the skin; a little 2% novocaine hydrochloride was injected subcutaneously. The needle of the infusion apparatus was inserted through the skin and pushed vertically down towards the artery. As soon as it entered the vessel blood could be seen gushing into the transparent tubing. The tip was pushed in a few mm. farther and a slow infusion of saline started to keep the needle patent. Records of the calf blood flow were made for about 10 min. A 3 min. infusion of one of the sympathomimetic drugs was then given. The rubber tubing was clipped to prevent blood gushing out of the the artery while the syringes were being changed. Records of the flow were made every ¼ min. during the infusion and every 2 or 3 min. for about 10 min. afterwards. Second and third infusions were sometimes given. The position of the needle in the artery was checked before it was taken out by allowing reflux of blood.

Intravenous infusions. In another series of experiments the solutions were given intravenously to see their effect on the heart as well as on the peripheral blood flow. The subject lay with his back raised slightly, covered with a blanket. A plethysmograph was fitted on the forearm. As the object of the experiments was to record the relative changes in blood flow occurring in 3 min. infusions it was considered unnecessary to keep the forearm at a strictly constant temperature throughout the experiment, so the water-bath was not used; instead the forearm was surrounded by a thick layer of cotton-wool (Barcroft & Edholm, 1946). This shortened the time taken to fit on the plethysmograph as the cuffs did not have to be cemented to the skin.

The infusion apparatus (Allen *et al.* 1946) consisted of a needle and a T-piece with one limb connected to a burette for sterile saline and the other for connexion to the 50 c.c. mechanically driven syringe containing one of the sympathomimetic substances. The apparatus was filled with sterile saline, the needle inserted into a vein near the antecubital fossa and the screw clip adjusted so that a trickle of saline flowed into the vein. Records of the blood flow were then made before, during and after several infusions as in the intra-arterial experiments. The order in which the drugs were given was varied.

In another series of experiments on the same subjects determinations of the arterial blood pressure were made on one arm by the auscultatory method before, during and after intravenous infusion of the drugs into the other.

The solutions of the sympathomimetic substances were made up from L-noradrenaline HCl (Tainter, Tuller & Luduena, 1948); L-adrenaline HCl (B.D.H.) and DL-N-isopropylnoradrenaline sulphate (Boehringer Sohn, Ingelheim a. Rh.)

RESULTS

Fig. 1 shows the results of the intra-arterial infusions, Figs. 2–4 those of the intravenous ones.

Two points need explanation:

(i) The rates of the intra-arterial infusions were less than those of the corresponding intravenous ones—otherwise the amount of the drug reaching the leg in unit time in the intra-arterial infusion would not have been equivalent.

(ii) In general, the rates of infusion of isopropyl were only about one-quarter those of noradrenaline or of adrenaline—otherwise, in the intravenous experiments, owing to the very powerful action of isopropyl on the heart, most subjects would have had distressing palpitation.

Blood flow in the forearm and calf. Fig. 1 shows that noradrenaline caused constriction. It did not do so during intravenous infusions (Fig. 2) for reasons explained later. Adrenaline caused a marked transitory vasodilatation whether it was given intra-arterially or intravenously (Figs. 1 and 3). Isopropyl also usually caused transitory dilatation (Figs. 1 and 4).

We must acknowledge that the resting forearm blood flows in nearly every experiment were definitely on the low side because the subjects were cold (see Allen *et al.* 1946, Figs. 3 and 4). If they had been warmer the changes in blood flow caused by the drugs would have been relatively the same but absolutely greater in c.c. per 100 c.c. forearm per min.

Arterial blood pressure. Fig. 2 shows that noradrenaline increased both systolic and diastolic blood pressures, confirming Goldenberg, *et al.* (1948). Infusions of adrenaline raised the systolic pressure, but the diastolic either was unchanged or fell slightly. (The initial fleeting drop of pressure of a few mm., described by Gordon & Levitt (1935) and confirmed by Allen *et al.* (1946), was not often recorded because it was not necessary in this research to take readings at $\frac{1}{4}$ min. intervals.) Isopropyl (Fig. 4) increased the systolic pressure and caused a marked fall in diastolic pressure.

Heart rate. Fig. 2 shows that noradrenaline usually caused bradycardia as found by Goldenberg *et al.* (1948) and by Duncanson, Stewart & Edholm (1949). Adrenaline (Fig. 3) caused tachycardia which subsided to some extent before the end of the infusion (Allen *et al.* 1946). Isopropyl caused tachycardia which showed no sign of subsiding during the infusion (Fig. 4).

Symptoms and signs. As a rule there were no symptoms with noradrenaline. The facial skin often became paler during the infusion and flushed afterwards.

Adrenaline often caused hyperventilation and palpitation; one subject felt tightness in the chest, and another a feeling of tiredness in the legs. There was usually facial pallor during the infusion and flushing afterwards.

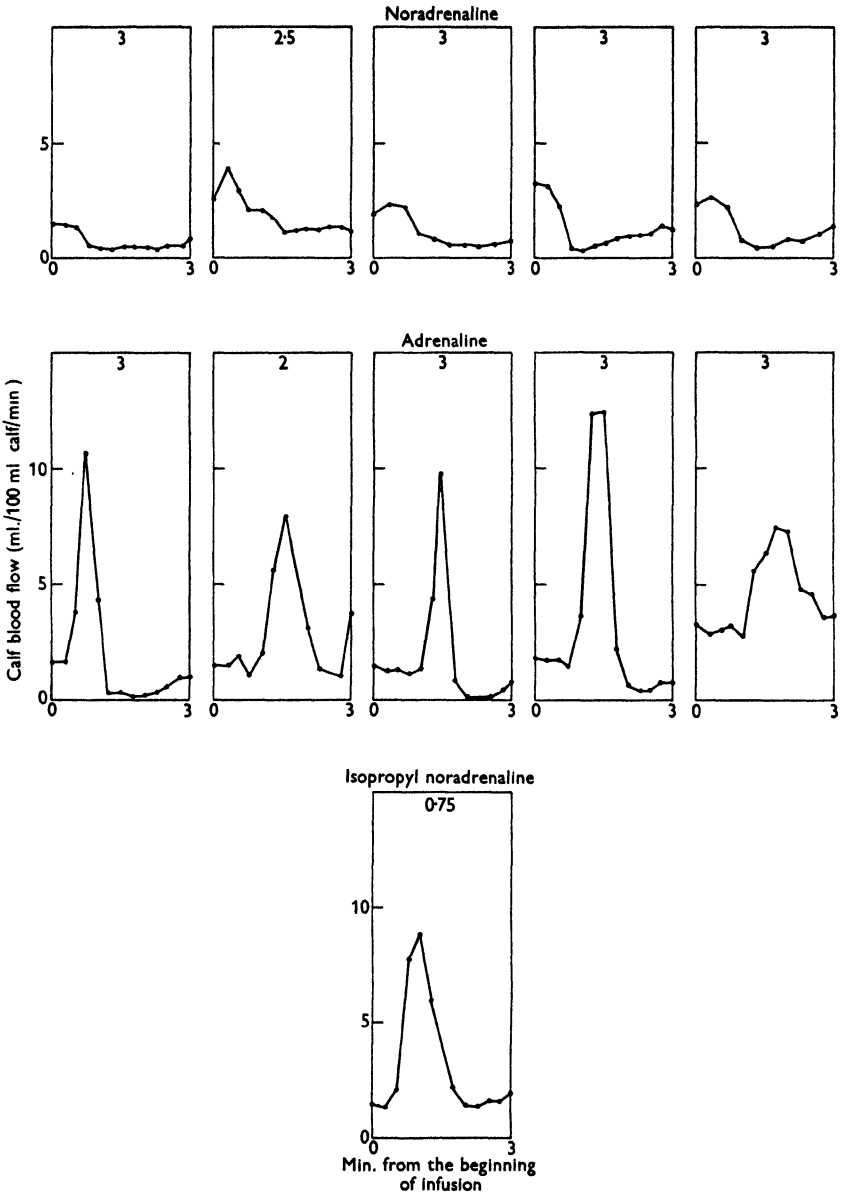

Fig. 1. Intra-arterial infusions of noradrenaline, adrenaline and isopropyl noradrenaline. Infusion began at time 0 and lasted 3 min. The rate, in μg./min. is given under the upper margin of each diagram. The first noradrenaline diagram, the first adrenaline one and the isopropyl one are experiments on the same subject. The other diagrams are from experiments on different subjects.

The diagrams for the last four infusions of adrenaline are from experiments in which a rubber tubing of rather large capacity connected the syringe to the intra-arterial needle. This explains the delay between the start of the infusion and the vasodilatation.

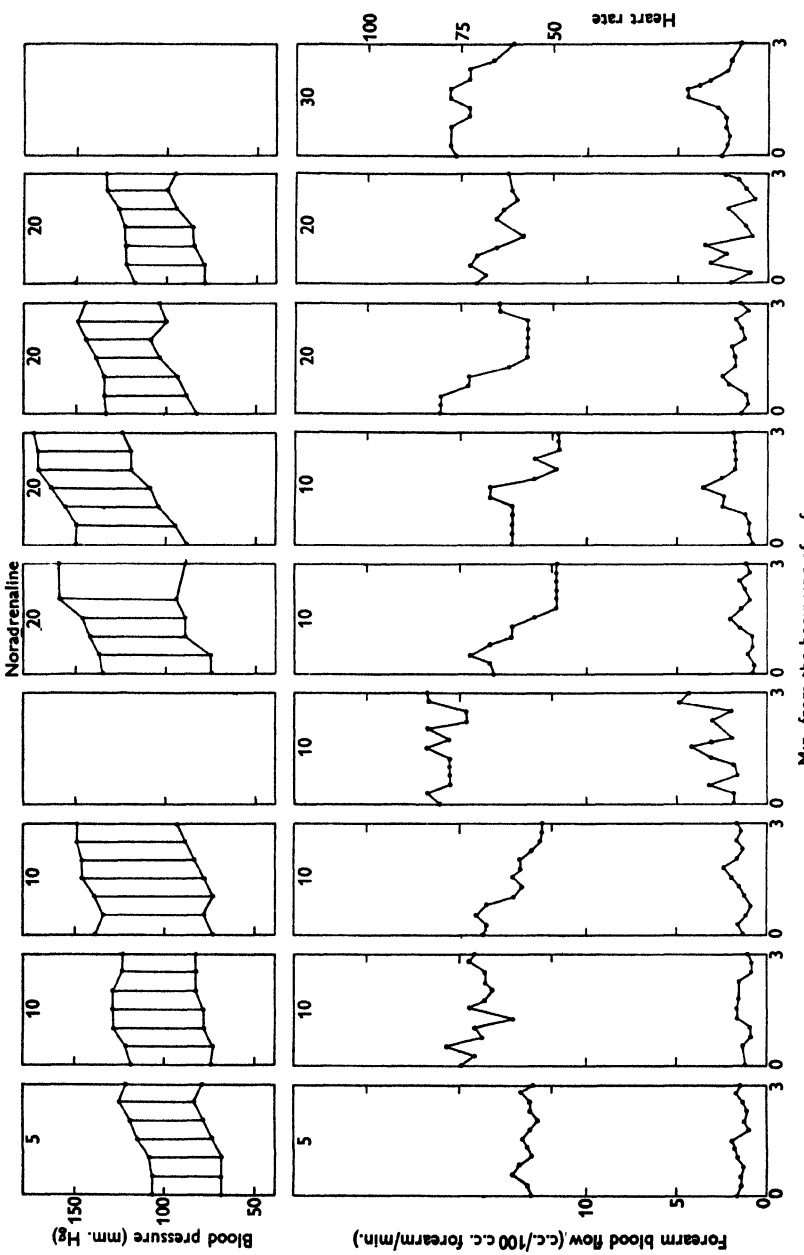

Fig. 2. Intravenous infusions of noradrenaline. Infusions began at time 0 and lasted 3 min. From above, arterial blood pressure, heart rate, blood flow. Each blood-pressure diagram and the diagram of heart rate and calf blood flow immediately below it are from experiments on the same subject on different days. Rate of infusion in μg./min. under the upper margin of each diagram.

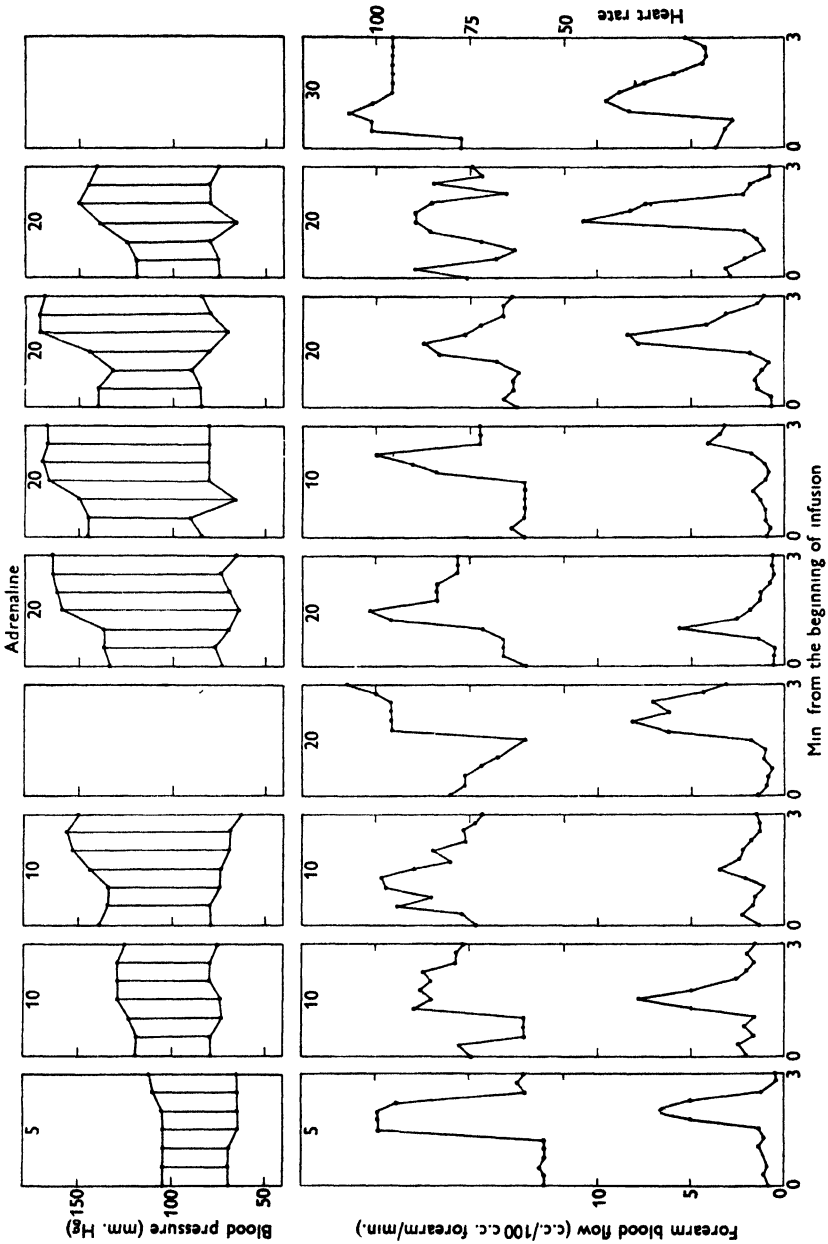

Fig. 3. Intravenous infusions of adrenaline. For further details see Fig. 2.

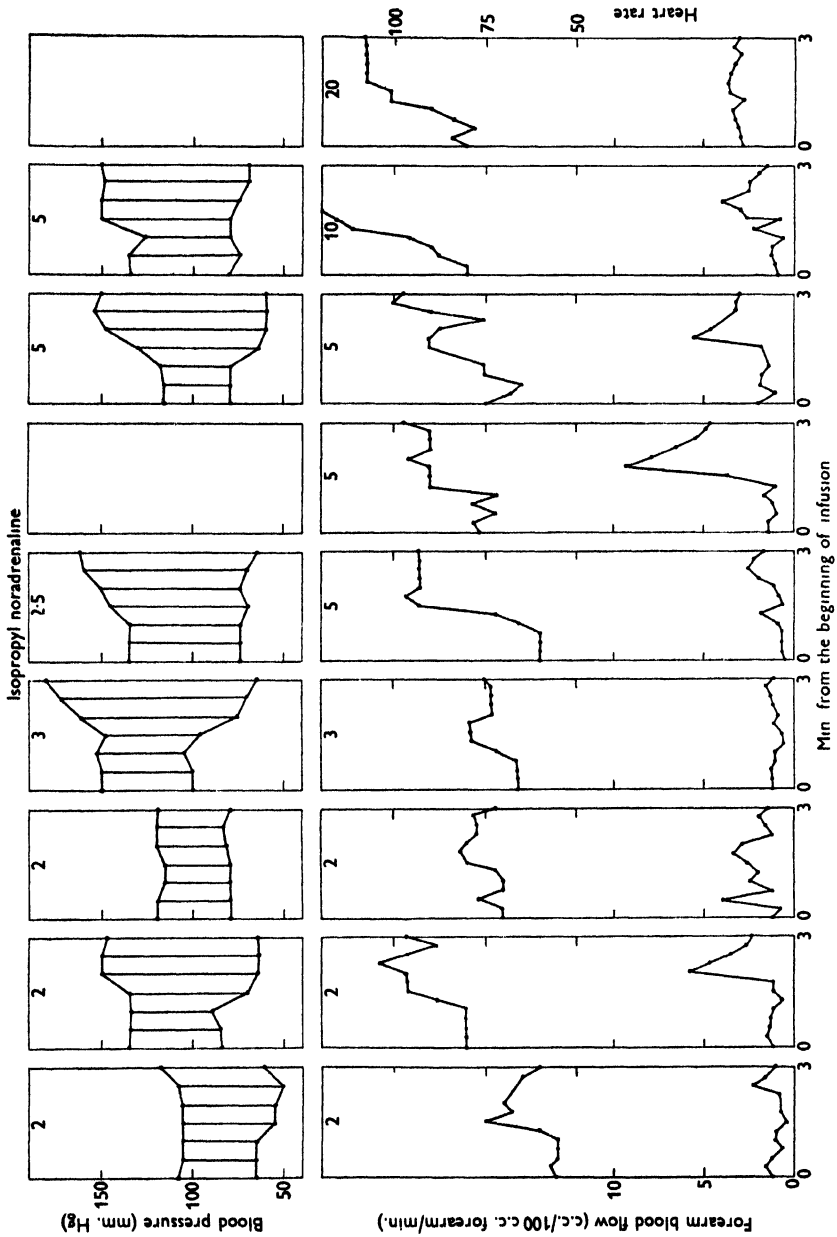

Fig. 4. Intravenous infusions of isopropylnoradrenaline. For further details see Fig. 2.

The symptoms with isopropyl were the same as with adrenaline except that palpitation was usually more marked and lasted to the end of the infusion. The skin of the face sometimes flushed and sometimes the carotid arteries pulsated visibly.

DISCUSSION

Blood flow in the calf and forearm. Grant & Pearson (1938) found that more than half the segment of the forearm enclosed in the plethysmograph was muscle. The amount of muscle in the calf is, of course, considerable, and in general these two parts of the body exhibit the same vascular response to any given procedure, a response which is often different from that given by the hand or foot where there is relatively much more skin. The blood flow in the calf and forearm is therefore generally considered to be mainly through the skeletal muscles.

The effect of intra-arterial injection of noradrenaline on the blood flow in the calf is to reduce it to about one-third (Fig. 1). Judging from the relative rates of blood flow to the skin and to the muscles in the forearm (Barcroft & Edholm, 1941) it seems likely that the reduction of the calf blood flow to one-third by noradrenaline was therefore probably mainly due to vasoconstriction in the skeletal muscles, and also to a small extent to cutaneous constriction.

It is interesting to note that Folkow, Frost & Uvnäs (1948) found that noradrenaline constricted both muscle and skin vessels in cat's limbs.

Fig. 2 shows that when noradrenaline was given intravenously there was no reduction in the forearm blood flow, if anything the reverse. Two factors probably explain this. First, the amount of noradrenaline reaching unit volume of forearm during intravenous infusion at 20 μg./min. was probably only about one-fifth of the amount reaching the same volume of calf during a 3 μg./min. intra-arterial infusion; its effect on the blood vessels in the forearm must therefore have been very feeble. Secondly, any vasoconstrictor effect there may have been would have been opposed by the tendency to passive dilatation due to the rise in the arterial blood pressure. Duncanson *et al* (1949) found that intravenous infusions of noradrenaline at 5 and 10 μg./min. caused a slight decrease in forearm blood flow, and a marked decrease in hand blood flow.

The experiments with isopropyl (Figs. 1 and 4) show that like adrenaline it caused a transitory dilatation in the calf. The infusions were too short for the blood flow to settle down to a steady rate. In the case of adrenaline Allen *et al.* (1946) gave intravenous infusions at 10 μg./min. for 10 min. and found that the flow subsided to about twice the resting rate (see Fig. 4 of their paper); probably in the isopropyl experiments described here the blood flow would have stabilized at something a little above the resting rate if the infusions had been longer. In the case of adrenaline, as probably with isopropyl, the transitory dilatation is in the muscles (Allen *et al.* 1946). It is independent of the sympathetic nervous system and its explanation is not known. Dilatation of capillaries followed by

contraction of arterioles is one possibility (Dale & Richards, 1918; Clark, 1933; Roome, 1938); another is suggested by the work of Straub (1907), namely, that it is due to a difference in the concentrations of adrenaline across the cell wall; this would be expected to be greatest at the beginning of the infusion and would tend to become less as adrenaline diffused in.

It is interesting to note that isopropyl causes vasodilatation in the gut and limbs (Konzett, 1940*b*), and in the lungs of dogs and cats (Hebb & Konzett, 1949).

Arterial blood pressure. Goldenberg *et al.* (1948) found that noradrenaline raised systolic, mean and diastolic pressures; adrenaline raised the systolic and the mean but had little effect on the diastolic pressure. Our results confirm this. Goldenberg *et al.* studied two most important factors determining the blood pressure—the cardiac output and the peripheral resistance. Noradrenaline either did not affect or decreased the output, and it also increased the peripheral resistance; this seems consistent with the fact that it raises both systolic and diastolic pressure. Adrenaline increased cardiac output considerably but caused an overall peripheral vaso-dilatation; this would explain why the diastolic pressure was not increased.

Isopropyl increased the systolic pressure but caused a marked fall in the diastolic. Its effect on the cardiac output and peripheral resistance in man is not known. The fact that it causes stronger palpitation than adrenaline, and a greater drop in the diastolic pressure suggests that there is an even greater increase in cardiac output with even more peripheral vasodilatation. If this is so it is odd that its vasodilator action in the limbs (Figs. 1 and 4) was no greater than that of adrenaline (Figs. 1 and 3), indeed probably not so great; it suggests that the main site of the vasodilatation was not in the skin or skeletal muscles.

Heart rate. It will be remembered that noradrenaline caused bradycardia, adrenaline caused tachycardia. This striking difference was first described by Goldenberg *et al.* (1948) and soon after independently by Barcroft & Konzett (1949). Its explanation is probably as follows. The direct actions of noradrenaline and of adrenaline on the heart rate have been compared on the isolated heart (frog, West, 1947; rabbit and cat, Ahlquist, 1948) and on the heart of the anaesthetized atropinized dog (Ahlquist, 1948); noradrenaline always induced tachycardia; but it acted much less powerfully than adrenaline. In man noradrenaline will probably excite the pacemaker, but much more feebly than adrenaline. In the normal subject the direct action of both drugs will be opposed by reflex vagal inhibition due to the rise in the arterial blood pressure. In the case of noradrenaline the reflex inhibitory action must predominate and so there is bradycardia, with adrenaline the direct excitatory action must predominate and hence tachycardia. This is supported by the fact that Goldenberg *et al.* (1948) found that noradrenaline bradycardia was abolished by previous atropinization. Moreover, in certain circumstances the inhibitory effect may

predominate with adrenaline too. For example, in man (Stacey, 1949) and in anaesthetized animals (Oliver & Schafer, 1894, 1895*a*, *b*) bradycardia occurs after a large intravenous injection.

We have not been able to find in the literature any mention of noradrenaline having caused bradycardia in animal experiments, possibly because they were not done with the object of studying the action of noradrenaline on the heart reflexes and so were performed on spinal animals (Barger & Dale, 1910; Stehle & Ellsworthy, 1937) after section of the vagi (Greer, Pinkston, Baxter & Brannon, 1938; West, 1947), or atropinization (Crimson & Tainter, 1939). In any case the effect of anaesthetics is not known and it would be interesting to see what noradrenaline does to the heart rate of anaesthetized man and of unanaesthetized animals.

Another question is why should the isopropyl tachycardia last to the end of the infusion while that due to adrenaline subsides to a large extent before it is over? This may be because of the different action of the drugs on the blood pressure; adrenaline increases it and so the reflex inhibition comes into play, isopropyl probably has very little action on the mean pressure so the heart rate is not restrained reflexly.

SUMMARY

1. The actions of noradrenaline and of isopropylnoradrenaline on the blood flow in the forearm and calf and on the heart rate and arterial blood pressure in unanaesthetized man have been examined and compared with those of adrenaline.

2. Noradrenaline causes: (i) constriction of the cutaneous vessels; (ii) constriction of the vessels in skeletal muscle (adrenaline causes transient vasodilatation); (iii) bradycardia (adrenaline causes tachycardia); (iv) rise in both systolic and diastolic pressures (adrenaline causes a rise in systolic pressure and either does not effect or causes a slight fall in diastolic pressure).

3. Isopropylnoradrenaline causes: (i) dilatation in the skin; (ii) transient dilatation in skeletal muscle; (iii) persistent tachycardia (adrenaline tachycardia subsides to a large extent during an infusion); (iv) rise in systolic and marked fall in diastolic blood pressures.

4. The differences in their actions are discussed.

We are most grateful to the subjects for their help; to Dr H. J. Swan, M.R.C.P., for valuable assistance; to J. Dalrymple for technical assistance; to Dr M. L. Tainter for the gift of L-noradrenaline to H. K.; to Dr Hildes for the loan of a mechanical infusion apparatus.

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MOTION PICTURE OBSERVATIONS ON OSTEOCLASTS *IN VITRO*

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The origin of osteoclasts is still an unsettled question; it seems at least possible that they may arise by fusion of monocytes or macrophages (Lacoste, 1923; Haythorne, 1929; Hancox, 1946). The latter group of cells possess a peripheral 'undulating membrane' demonstrable by dark-ground cinemicrography (Carrel & Ebeling, 1926). Time-lapse motion picture records of osteoclasts in tissue culture were therefore undertaken to ascertain whether osteoclasts are similarly endowed.

METHODS

Small fragments of chick embryo frontal bones of 14 days' incubation were explanted as hanging drop cultures according to the technique already described, and, as observed previously, osteoclasts wandered out from the fragments (Hancox, 1946). Suitable preparations were mounted in a plasma wagulum between cover-slips and photographed continuously by a time-lapse microcinegraphic apparatus for periods of up to 6 hr., and were thereafter discarded in view of the possibility of degenerative changes.

RESULTS

On projection of the films it can be seen that the peripheral zone of cytoplasm of the cells frequently assumed an appearance corresponding to the description given by Carrel & Ebeling (1936) of the 'undulating membrane' of the monocyte. That is, there appeared quite suddenly a flashing, brightly illuminated line, apparently the border of a swiftly moving zone of fluid ectoplasm. The movement of this line was sometimes reminiscent of the edge of a curtain disturbed by a breeze, and sometimes of the activity of a long flagella-like process. This appearance was present intermittently rather than continuously, and it is of interest that it was encountered on the edges of fine cytoplasmic filaments. It is, of course, not possible to illustrate this membrane in 'stills' obtained by enlargements of single frames of the cine-film.

Osteoclasts have a tendency to form bilobed masses in tissue culture (Hancox, 1946) and in the intact animal (Barnicot, 1947). Sometimes the masses are

united by a thin cytoplasmic thread; at others, they are separated, but bear fine-pointed filaments directed towards one another which suggests that large osteoclasts may produce smaller individuals by fission. A record was obtained of the production of such a fine, long filament (Pl. I, figs. 1–6). The fine waist attained a length of at least 100 μ. and its edge 'undulated'. It was not observed to break.

DISCUSSION

These facts have a possible application to the controversy regarding the origin of the cells. In the first place, their possession of an undulating membrane seems to afford further support to the view that osteoclasts form by fusion of wandering cells, which are similarly endowed, rather than from connective tissue cells, such as osteoblasts, which are not. In the second place, the ability of the cells to form long filaments may be of importance. The hypothesis of an origin from connective tissue cells depends to a large extent upon interpretation of sections of fixed material. Apparent cytoplasmic continuity between osteoclasts and their supposed precursors, or their mutual propinquity, seem to be the chief criteria adopted. However, it is possible to imagine that as the osteoclast moves through the tissues, it could form a long filament; this, as it formed, would touch upon other cells of different nature. Microscopic examination of tissue sections of fixed material would offer every possibility of misinterpretation; the osteoclast would appear as if in continuity with, and hence to be forming from, individuals which chance alone dictated should be nearby.

The flagella-like appearance of the undulating membrane encountered from time to time demands further investigation, and for this purpose, the phase-contrast microscope would be the method of choice.

SUMMARY

Osteoclasts possess a peripheral undulating membrane in tissue culture, and form fine cytoplasmic filaments. These facts support the hypothesis that osteoclasts are related to wandering cells rather than to osteoblasts.

This work was undertaken with the assistance of a grant from the Joint Research Committee of the University of Liverpool; it was carried out in the laboratory of SIMPL Ltd., London, where the kinemicrographic apparatus is situated. Thanks are due to R. McV. Weston for the motion picture records and the photographic enlargements.

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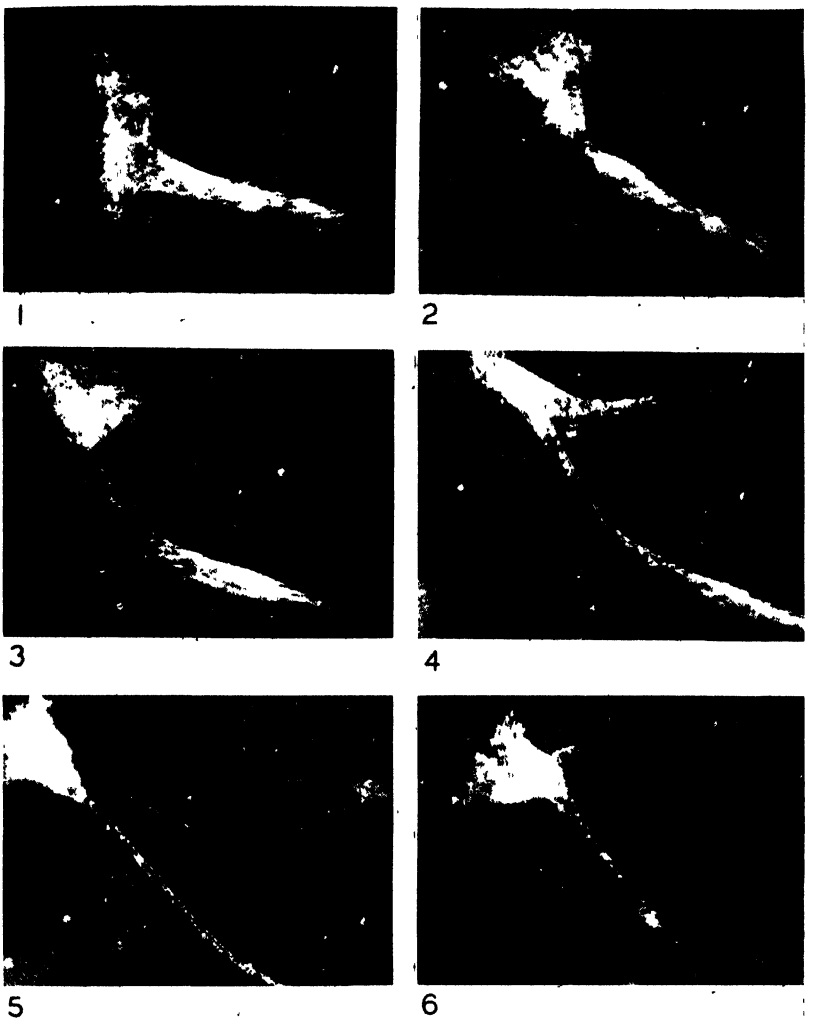

The photographs are enlargements of the original 16 mm. film record; the magnification is approximately ×670.

Fig. 1. An osteoclast showing granular cytoplasm. No intracellular details are visible.

Fig. 2. Same cell, 22 min. later. A waist is beginning to form.

Fig. 3. Same cell, 25 min. later. Two well-defined lobes are now present.

Fig. 4. Same cell, 35 min. later. The cytoplasmic filament connecting the two lobes shows clearly; the upper lobe has thrown out a pseudopod.

Fig. 5. Same cell, 35 min. later. The lower lobe has moved out of the field. Granules can be seen within the filament.

Fig. 6. Same cell, 30 min. later. The field was moved to bring the upper lobe back into the picture; meantime the lower lobe had moved even farther away.

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THE INFLUENCE UPON THE VITAL CAPACITY OF PROCEDURES CALCULATED TO ALTER THE VOLUME OF BLOOD IN THE LUNGS

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Alterations in the vital capacity (v.c.) in diverse circumstances have been interpreted as due to increase or decrease in the amount of blood in the thoracic cavity. There is, however, no satisfactory agreement upon the means whereby pulmonary congestion may limit the v.c., and the mere act of recording the v.c. is likely to alter the volume of blood in the lungs. A comparison has been made between alterations produced by collection of blood elsewhere in the body, and those produced by exposing the lungs themselves to positive or negative pressures. Evidence has thus been obtained both upon the regulation of pulmonary blood volume and upon the means whereby alterations in this volume affect the v.c.

SUBJECTS AND METHODS

The author was the subject for most of the experiments; a few other healthy male subjects were also used.

The v.c. was recorded with a Krogh spirometer and measured to the nearest 10 c.c. All volumes are expressed at 37° C. saturated.

Except where otherwise stated, the subject was supine on a flat level couch, and rested for at least 15 min. in this position before experiments started.

For administration of amyl nitrite the circuit shown in Fig. 1 was used. The ampoule was crushed through the flexible respiratory tubing, and by turning tap *A* the subject could inhale fresh air or amyl nitrite without the airtight spirometer circuit being opened. The crushing of ampoules of amyl nitrite within the closed circuit caused no detectable change of volume.

For taking single inspirations at negative pressure the circuit shown in Fig. 2 was used. By turning taps *X* and *Y* the subject was caused to inspire freely through the airway *A*, or under negative pressure through the 20 cm. water-seal *B*, whilst volumetric recordings continued. The pressure recorded on the manometer between mouth and water-seal while the subject was inspiring through *B* was considerably below -20 cm. H_2O, and when subjects inspired rapidly it was often -40 cm. H_2O.

For continuous negative pressure respiration, air was rebreathed from a metal drum of about 50 l. capacity, fitted with a CO_2 absorbent and continuously evacuated by a filter pump, with a variable water seal to admit air if the pressure fell too low. In this way a subatmospheric pressure was continuously maintained, with a respiratory fluctuation of about 20 mm. Hg recorded by

a Hg manometer between mouthpiece and drum. A 3-way tap near the mouthpiece served to connect the subject to the spirometer after a period of negative pressure breathing.

Positive pressure breathing was achieved by connecting the subject to a weighted bag, with added oxygen and a CO_2 absorbent for rebreathing experiments.

The conventional meanings of 'significant' ($P<0{\cdot}05$) and 'highly significant' ($P<0{\cdot}01$) have been used.

RESULTS

Venous congestion. It has been shown by many workers, e.g. Hamilton & Morgan (1932), Asmussen, Christensen & Sjöstrand (1939) and Sjöstrand (1941) that the v.c. is increased if blood is accumulated in the legs by means of

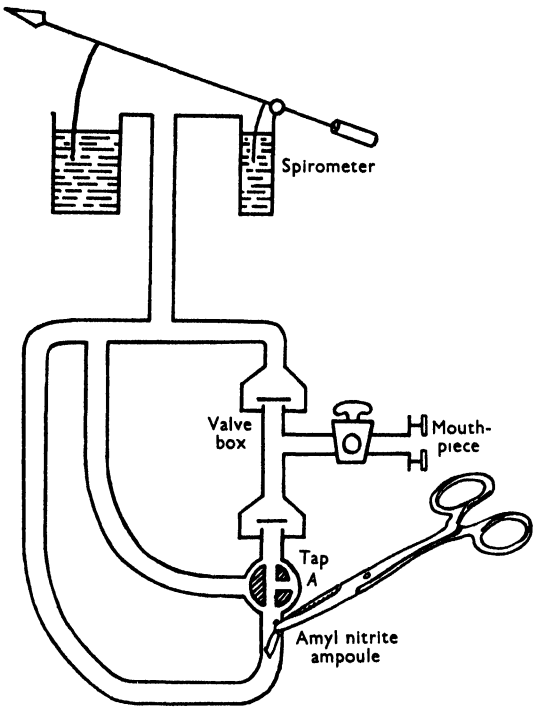

Fig. 1. Spirometer circuit used to investigate the effect of amyl nitrite upon the vital capacity.

pneumatic cuffs encircling the thighs at about venous pressure. In the present investigation it was found that the v.c. could be regularly increased if the cuffs were applied at a pressure of 90 to 100 mm. Hg for 15 min. with the subject erect, and the v.c. then measured with the subject recumbent. Subjects R.L. and G.C. showed increases of 320 ± 96 c.c. (S.E. of difference of means of 10 control and 5 congestion experiments) and 321 ± 112 c.c. (11, 4), and the mean fall of v.c. 1 or 2 min. after readmitting blood in twenty-six experiments on J.N.M. was 470 ± 70 c.c.

Position. It seems generally accepted (Hamilton & Morgan, 1932; McMichael & McGibbon, 1939; Sjöstrand, 1941) that the increased v.c. on standing is due

to diminution of blood in the pulmonary vessels. Table 1 shows the means of ten determinations erect and ten supine, on each of 6 days. On 2 days there was no significant difference, on the other 4 days a highly significant difference.

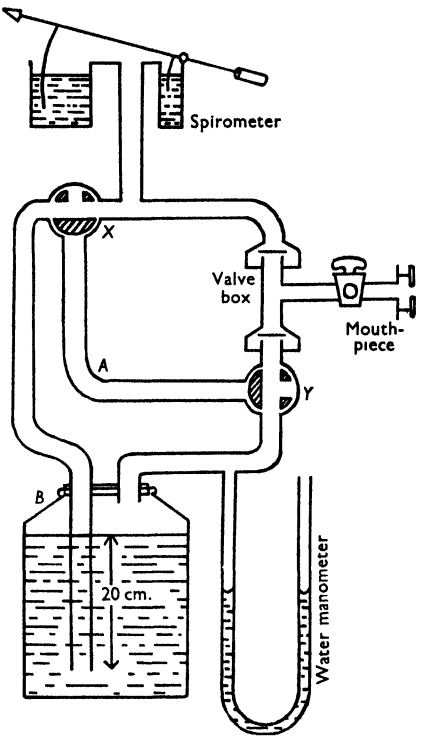

Fig. 2. Spirometer circuit used in investigating the effect of negative pressure inspiration upon the vital capacity. *A*, airway for inspiration at atmospheric pressure; *B*, water seal for inspiration at negative pressure; *X*, *Y*, taps whereby subject can be connected to one or other inspiratory airway.

TABLE 1. Means of ten determinations of v.c. in c.c., supine and erect, with S.D. of individual values, difference of means and S.E. of difference, and *P*, the probability that this difference would be found by chance. Subject J.N.M.

Day	Supine	Erect	Difference	*P*
1	5465 ± 41	5590 ± 91	125 ± 31	<0·01
2	5515 ± 82	5514 ± 77	−1 ± 36	>0·9
3	5452 ± 70	5424 ± 74	−28 ± 32	0·3–0·4
4	5380 ± 117	5524 ± 100	144 ± 48	<0·01
5	5446 ± 130	5662 ± 66	216 ± 46	<0·01
6	5503 ± 31	5622 ± 72	119 ± 24	<0·01

Amyl nitrite. The inhalation of amyl nitrite has been used by Sjöstrand (1941) to withdraw blood from the pulmonary into the systemic circuit, and upon two subjects he produced in this way increases of 170 and 230 c.c. in the v.c. The same technique has been used upon J.N.M., with similar results.

After an initial recording of the v.c., and 45 sec. of quiet rebreathing, a 3 minim ampoule of amyl nitrite was crushed; the subject took a few deep breaths to absorb the drug as rapidly as possible, and 30 sec. later tap *A* (Fig. 1) was turned to enable him to inspire fresh air for 15 sec. before the final recording of the v.c. The timing was chosen empirically as that which produced the largest change in v.c., and at the time of the second v.c. the subject was always rather faint and very flushed.

Control experiments without amyl nitrite were also performed, in order to find out how much air was lost from the spirometer by respiratory exchange between the 2 v.c. determinations.

In 17 experiments, the mean increase of v.c. after inhaling amyl nitrite was 238 ± 66 c.c. The drop in spirometer volume from the first to the second maximal expiration was 178 ± 28 c.c., a value very similar to that found in controls, whilst the drop from the first to the second maximal inspiration was 415 ± 71 c.c., much more than the loss ever found in controls. It appears that the rise of v.c. produced by amyl nitrite was mainly due to an increased ability to inspire.

Negative pressure respiration. It was hoped that by breathing air at below or above ambient pressure the volume of blood in the pulmonary vessels might be increased or decreased and the v.c. thereby lowered or raised. Respiratory movements might also, however, be mechanically impeded or assisted. Paton & Sand (1947) state that the v.c. is reduced when breathing air at either positive or negative pressure, and a few experiments upon J.N.M. suggest that maximal inspirations against a water seal 20 cm. deep may be restricted to the extent of 300–500 c.c., whilst maximal inspirations at a pressure of 35–40 mm. Hg may be 300 c.c. deeper than those from air at ambient pressure. It is thus desirable that air at ambient pressure should be breathed when the v.c. is being measured, despite the danger that the change to ambient pressure may shift blood to or from the pulmonary bed.

To measure the v.c., therefore, after 1 min. negative pressure breathing, the subject inspired maximally, completed his inspiration from room air with or without a brief respiratory relaxation, and expired maximally into the spirometer. The interval from turning over to ambient pressure to completion of inspiration was measured on a fast drum on seven occasions and was found to be 0·6–1·2 sec., but a further 5 or 10 sec. elapsed before completion of expiration. This procedure lowered the v.c. by 100–300 c.c., as shown in Table 2. In other experiments, also shown in Table 2, the subject breathed room air quietly for 5 sec. or more after a minute's exposure to negative pressure, and the v.c. had by then always returned to normal. When the inspiratory v.c. was measured immediately after a minute's negative pressure breathing it was the same as in controls (difference $= 87 \pm 91$ c.c. (7, 8)). Maximal expiration was complete within 1–3 sec. of release from negative pressure, and maximal inspiration within 6–8 sec. It appears that negative pressure breathing,

presumably by accumulating blood in the lungs, decreases the v.c. subsequently determined at ambient pressure, that the v.c. returns to normal within 5 sec., and that the reduction is due to limitation of inspiratory ability.

In other experiments, upon six subjects, the v.c. was measured after a single maximal inspiration against negative pressure, completed at atmospheric pressure as before; the fall was now smaller, as seen in Table 3, and was often non-significant.

TABLE 2. Means of ten determinations of v.c. in c.c., before and after 1 min. negative pressure respiration, with S.D. of individual values, and difference from control with S.E. of difference of means. Subject J.N.M.

Day	Pressure, mm. Hg Inspiration	Pressure, mm. Hg Expiration	Control v.c.	Sec. after negative pressure	V.c.	Difference of means
1	−17	−2	5379 ± 90	—	5158 ± 74	−221 ± 37
2	−32	−12	5261 ± 84	—	5040 ± 82	−220 ± 37
3	−42	−18	5278 ± 123	—	4970 ± 252	−309 ± 89
4	−40	−20	5324 ± 59	—	5154 ± 77	−170 ± 31
*5	−50	−30	5317 ± 129	—	5042 ± 104	−274 ± 68
—	—	—	—	5	5219 ± 86	−98 ± 63
—	—	—	—	10	5229 ± 45	−88 ± 56
—	—	—	—	20	5253 ± 64	−64 ± 59
—	—	—	—	30	5251 ± 32	−66 ± 54
6	−60	−40	5164 ± 104	—	5044 ± 102	−120 ± 46
—	—	—	—	5	5152 ± 77	−12 ± 41
—	—	—	—	30	5195 ± 97	+31 ± 45

* Figures for this day are all means of six determinations.

TABLE 3. Mean v.c. in c.c. after inspiration at ambient pressure or against a water-seal 20 cm. deep, with S.D. of individual values, and number of observations in brackets, and difference from control with S.E. of difference of means

Subject	Control v.c.	V.c. after negative pressure inspiration	Difference of means
J.N.M.	5044 ± 86 (10)	4917 ± 159 (10)	126 ± 57
J.N.M.	5018 ± 59 (10)	4964 ± 69 (10)	54 ± 29
J.N.M.	4848 ± 149 (10)	4784 ± 87 (10)	65 ± 55
Ra	4679 ± 65 (10)	4617 ± 42 (11)	61 ± 24
Ra	4902 ± 64 (10)	4857 ± 87 (10)	45 ± 34
Sp	4800 ± 204 (18)	4907 ± 176 (18)	−106 ± 64
A.H.D.	4431 ± 98 (10)	4418 ± 127 (11)	13 ± 50
L.W.	4135 ± 274 (10)	4144 ± 161 (10)	−9 ± 100
Gr	3225 ± 166 (16)	3240 ± 141 (16)	−15 ± 52

Some indication that the presumed action of negative pressure inspiration in aspirating blood into the thorax operates throughout, and not only at the end of, an inspiration was obtained by a comparison between the amount of air expirable after either taking a full breath against negative pressure, or taking the greater part of a breath at ambient pressure and completing it against negative pressure. The volume was greater with the latter procedure by 67 ± 38 (10, 11) and 75 ± 33 c.c. (10, 10) upon subject Ra and by 141 ± 97 c.c. (10, 10) upon J.N.M.

Positive pressure respiration. When after a full expiration the lungs had been distended at a pressure of 30–45 mm. Hg, and the mouthpiece was connected to the spirometer, very little air entered the spirometer, and it appeared that the glottis must be firmly closed. If then maximal expiration was made into the spirometer, and the volume of gas emerging was corrected for expansion due to the fall of pressure, it was found that under a positive pressure of 35–39 mm. Hg the lungs held 302 ± 47 c.c. (10, 10) more than at atmospheric pressure. The residual air, a necessary figure for this calculation, has been assumed to be 25 % of the v.c. (Hurtado & Boller, 1933), a figure which agrees well with determinations made upon the author some years ago; small differences in this volume make very little difference to the calculation. Either under this positive pressure the lungs at full inspiration contain 300 c.c. of blood less than at ambient pressure, or the thoracic cage has been expanded more than is possible by muscular effort. This latter effect can best be eliminated if, after positive pressure inspiration, a brief expiration to and maximal inspiration

TABLE 4. Mean v.c. in c.c. after full inspiration at either ambient pressure or positive pressure of 31–34 mm. Hg, followed by rapid expiration and maximal inspiration at ambient pressure as initial phase of the v.c. recording. S.D. of individual values inserted, with number of observations in brackets, and difference from control with S.E. of difference of means. Subject J.N.M.

Preliminary period on positive pressure	Control v.c.	V.c. after positive pressure inspiration	Difference of means
One inspiration	4784 ± 39 (10)	4786 ± 113 (9)	2 ± 38
One inspiration	4895 ± 86 (10)	4959 ± 59 (10)	65 ± 33
One inspiration	5000 ± 118 (10)	5039 ± 153 (10)	39 ± 61
½ min.	5083 ± 73 (10)	5161 ± 116 (10)	78 ± 44
½ min.	5216 ± 142 (10)	5166 ± 142 (9)	−50 ± 65

from air are made before expiring fully to measure the v.c. This procedure must be very rapid if reaccumlation of blood in the lungs is to be avoided. Recordings were therefore made upon a fast drum, on which time intervals could be estimated to about 0·1 sec. It seemed possible that the second, very hurried, maximal inspiration might not be as deep as a more leisurely effort, so in control experiments a rapid shallow expiration and maximal inspiration intervened between the first full inspiration and the maximal expiration. In many such series of control experiments the second maximal inspiration fell short of the first by 100–200 c.c., and the control v.c. was thus lower than usual.

In some early series of experiments a single maximal inspiration at 30–45 mm. Hg. positive pressure produced a highly significant mean increase of v.c. of from 104 to 163 c.c. in series of 10–25 experiments. In more recent experiments with a fast drum and accurate observation of the pressures used no significant change in v.c. has ever been observed, after either a single breath or ½ min. quiet respiration at a positive pressure between 31 and 34 mm. Hg, as will be seen from Table 4. The time elapsing between the opening of the glottis which let out air into the spirometer and the completion of the subse-

quent maximal inspiration at ambient pressure varied between 0·3 and 0·7 sec. in the 30 experiments in which it could be accurately measured, and there was no correlation ($r = 0{\cdot}116$) between time interval and v.c. such as would be expected if the time over this range were critical. A variety of other techniques, such as longer periods of positive pressure breathing and release of pressure at the end of expiration before v.c. determination by a maximal inspiration, have also failed to produce any significant change in v.c. It seems that if positive pressure breathing alters the v.c., the change is reversed within 0·3 sec. of changing over to ambient pressure, a period too short to allow the shift of any considerable volume of blood into the lungs.

The nature of the circulatory response to positive pressure breathing was not investigated, but that it is very effective was demonstrated by continuing for 5 min. to breathe air at a pressure of 33–34 mm. Hg above atmospheric.

DISCUSSION

The alterations of v.c. produced by venous congestion of the legs, posture, and amyl nitrite merely confirm the work of other authors, and to alterations in pulmonary blood volume have been ascribed the changes in v.c. produced by haemorrhage (Glaser & McMichael, 1940), in left-sided heart failure (Hochrein & Keller, 1934), on immersion in water (Hamilton & Mayo, 1944), and after administration of adrenaline (Hochrein & Keller, 1934; Sjöstrand, 1941). More surprising is the great difficulty encountered in altering the v.c. by negative or especially positive intrapulmonary pressures. This might be held to support the conception of the reservoir function of the lungs (Sjöstrand, 1941), and to indicate very efficient adaptation to altered intrapulmonary pressures.

Pulmonary inflow is an active process, depending upon the contractile force of the right ventricle, and so might more readily adapt itself to the restraint imposed by a positive intrapulmonary pressure, whereas pulmonary outflow, which is probably mainly passive, might less readily adapt itself to the restraint of a negative intrapulmonary pressure. Cournand, Lauson, Bloomfield, Breed & Baldring (1944) have shown by cardiac catheterization that whereas the human right ventricle normally develops a systolic pressure of 20–30 mm. Hg, pressures of 80 mm. Hg and over can be developed in disease. The effectiveness of such adaptation is shown by the possibility of breathing air at a pressure of over 30 mm. Hg for 5 min. without discomfort.

The mechanism whereby alteration of pulmonary blood volume alters the v.c. is obscure, and there seems to be no good reason for equating changes of v.c. volumetrically with changes of pulmonary blood volume, as Sjöstrand (1941) appears to do. If we neglect the inspiratory accumulation of blood within the thorax (Lewis, 1908; Katz & Gauchat, 1924; Heinbecker, 1927; Cahoon, Johnson & Michael, 1940; Shuler, Ensor, Gumming, Moss & Johnson, 1942; Battro, Segura, Elicabe & Araya, 1944) and suppose that respiratory movements

remain unrestricted, then the addition of a given volume of blood to the lungs would lower the residual air and total lung volume by that amount, but would leave the v.c. unaltered. If the v.c. is altered, then either the respiratory movements are restricted or facilitated, or the inspiratory accumulation of blood is altered. On the latter assumption, since the v.c. can vary over a range of $\frac{1}{2}$–1 l. with different pulmonary blood volumes, we should have to assume that the volume of blood accumulating in the lungs during one maximal breath can vary over this surprisingly large range.

The positive pressure experiments can be used as evidence against any large inspiratory accumulation of blood in the lungs. Although the right ventricle can maintain its output against a pressure of over 30 mm. Hg, it seems likely that a single pulmonary inflation at this pressure would prevent the normal purely passive inspiratory blood accumulation. Apart from any possible overdistension of the thorax, the v.c. should therefore be increased by an amount equal to the volume of blood whose entrance to the thorax has been prevented. In fact, the v.c., measured 0·3–0·7 sec. after release of the pressure, is usually unaltered, and the timing between 0·3 and 0·7 sec. is not critical. The period of 0·3 sec. could include only one ventricular systole with ejection of, say, 100 c.c. blood, or might occur entirely during diastole. The scatter of v.c. determinations does not preclude an increase of up to 100 c.c. with positive pressure inflation, but it does not appear that the normal accumulation of blood in the lungs during a deep inspiration can be much more than this; thus it seems that the larger increases of v.c. produced by, for instance, amyl nitrite, must be due to a greater amplitude of respiratory movements.

It must be supposed from those experiments where the interrupted inspiration technique was omitted that a positive pressure of 35–40 mm. Hg was capable of overdistending the chest by some 300 c.c., which is actually a smaller increase than would be supposed from the data of Rahn, Otis, Chadwick, & Fenn (1946). In other words, the v.c. is limited by the power which can be exerted by the inspiratory muscles, and not by any rigid anatomical limit of chest size.

The trifling change in v.c., when air is inspired at a negative pressure and the inspiration is completed rapidly at ambient pressure, similarly suggests that under negative pressure not much extra blood is aspirated into the chest during one inspiration. The definite fall in v.c. after 1 min. of negative pressure breathing, and the recovery within 5 sec., indicate that under these conditions blood can be accumulated in the pulmonary vessels, but is rapidly passed on when ambient pressure is restored. The subjective discomfort in these experiments was much greater than with positive pressure breathing, and may have been a consequence of pulmonary congestion.

The experiments with amyl nitrite and negative pressure suggest that decrease or increase of volume of blood in the lungs alter v.c. by facilitating or

impeding inspiration, leaving the residual air volume relatively unaltered, which agrees with the findings of Hamilton & Morgan (1932), Asmussen *et al.* (1939), and Glaser & McMichael (1940). The alterations might be due to a reflex limitation of respiratory movements, as suggested by Hamilton & Morgan (1932), or to an alteration in the physical properties of the lungs when congested with blood (Christie & Meakins, 1934; Mack, Grossman & Katz, 1947).

SUMMARY

1. Accumulation of blood in the legs increases the vital capacity (v.c) by $\frac{1}{4}$–$\frac{1}{2}$ l., inhalation of 3 minims of amyl nitrite by about $\frac{1}{4}$ l., and assumption of the erect posture by a smaller amount.

2. The increase with amyl nitrite is due to an increased depth of inspiration.

3. A single inspiration at a pressure of up to 30 mm. Hg below or 35 mm. Hg above ambient produces little or no change in the v.c. if the inspiration is rapidly completed at ambient pressure before expiration. Longer periods of positive pressure breathing are equally without effect, but 1 min. of negative pressure breathing lowers the v.c. by up to 300 c.c. The reduction is due to a limitation of inspiratory power, and has disappeared after 5 sec. breathing at ambient pressure.

4. A single maximal inspiration at 35–40 mm. Hg positive pressure admits to the lungs 300 c.c. more air than does a maximal inspiration at ambient pressure. Reasons are given for supposing that this is due to overdistension of the thorax rather than to expulsion of blood.

5. Reasons are given for supposing that a normal maximal inspiration does not aspirate more than about 100 c.c. of blood into the lungs, and that alterations of v.c. due to alterations of pulmonary blood volume are due to limitations of the amplitude of the respiratory movements and not to variations in the amount of blood aspirated into the lungs in the course of the v.c. determination.

My thanks are due to those who have acted as subjects, and to Mr S. Langford for technical assistance.

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THE RATE OF DISCHARGE OF THE EXTRAOCULAR MOTONEURONES

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Cooper & Eccles (1930) found that the duration of the isometric twitch of the internal rectus of the cat is short, and the rate of stimulation required to produce a fused tetanus is high (350/sec.). Muscles with greater contraction times have correspondingly lower fusion frequencies, being of the order of 30 and 100/sec. for the soleus and gastrocnemius respectively. Numerous workers have observed the rate of discharge to various muscles of the limbs during reflex contraction in animals (Adrian & Bronk, 1929; Denny-Brown, 1929), and during voluntary movement in man (Smith, 1934; Lindsley, 1935; Gilson & Mills, 1941; Weddell, Feinstein & Pattle, 1944); but the rate of discharge of the extraocular motoneurones does not appear to have been studied.

METHODS

Observations were made on ten cats and two goats. Two of the cats were anaesthetized with chloralose (0·08 g./kg.), and the remainder were 'decerebrated' by the trephine method usually in front of the colliculi so as to leave intact the third nucleus, the pupils remaining small. One or both common carotid arteries were left untied, and in experiments where extensive dissection of the orbit was necessary the left trigeminal nerve was divided inside the skull. Sometimes it was necessary to quieten such 'high-section' decerebrate animals with one-quarter the anaesthetic dose of nembutal (pentobarbital sodium). Impulses were picked up from the muscles by 38 s.w.g. constantan wires twisted together, varnished with shellac, baked, and cut across obliquely. Such electrodes after insertion into the muscle were tied to its tendon. For single fibre preparations of the nerve to the inferior oblique the roof of the orbit, the zygoma and coronoid process of the mandible were removed. The eye was eviscerated and retracted forward and medially. The nerve was then identified and its sheath removed. It was subdivided until only a single unit appeared to be active when it was placed on twin platinum electrodes held in a support which was screwed to the skull. The upper portion of the animal was kept in a screened warmed, moist chamber. Stimulation was brought about by appropriate movement of the head as described later, or by galvanic stimulation of a labyrinth, one electrode being placed in the external auditory canal and the other under the skin of the chest wall. The electrodes were connected in series with a variable resistance of 10,000 Ω. to a 12 V. battery. The two goats which had been prepared for another purpose

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(Cooper, Daniel & Whitteridge, 1949) were anaesthetized with nembutal, and wire electrodes were inserted into the lateral rectus.

The action potentials were recorded by the usual amplifier, cathode-ray tube and camera. The output of the amplifier was also fed to a loud-speaker.

RESULTS

Impulses arising peripherally and probably caused by injury from the inserted electrodes

It was surprising how readily impulses of very high frequency were recorded from wire electrodes present in the muscle. These, however, were not the result of reflex stimulation but appeared to be due to the presence of the electrode inside the muscle. Various observers (Weddell, *et al.* 1944) have described short-lived 'insertion potentials', but otherwise it is generally agreed that impulses are not recorded from relaxed muscles (Adrian & Bronk, 1928; Smith, 1934; Lindsley, 1935; Gilson & Mills, 1941; Hoefer, 1941). These impulses which are now described were, however, obtained not only on insertion of the electrodes but appeared to arise spontaneously. Of course, slight movements within the muscle relative to the electrode could not be excluded. Such impulses were recognized to be peripheral in origin, because similar patterns of activity could be picked up from muscles whose nerve supply (the third nerve) had been divided some 3 hr. previously, and when present in innervated muscle they could not be inhibited by the appropriate movement of the head. The patterns of activity closely resembled the three types described by Adrian (1930) for injured mammalian nerve. They consisted of: (i) a sustained burst beginning at a frequency often greater than 200/sec. and lasting several seconds; or (ii) short bursts from 0·1 to 1 sec. in duration and repeated at intervals of the same order, the frequency varying from about 50 to over 200/sec.; or (iii) irregular and slow (less than 20/sec.) impulses during which bursts of two, three or four impulses may sometimes be seen. Actually there were gradations between these types, and a high-frequency burst sometimes subsided over several seconds to a slow irregular pattern. As in the activity described by Adrian, the intervals between successive spikes progressively increased from the commencement of a burst, a further point of distinction from reflex activity in which the spike intervals at first progressively decreased.

Fig. 1 illustrates this kind of activity. The parts A 1, A 2 and A 3 show action potentials picked up from the left superior rectus of a decerebrate cat in which the section was below the third nucleus and the third nerves were divided 3 hr. previously. In A 1 a burst begins at a rate of about 175/sec. and decreases to a rate of about 100/sec. before ceasing. In A 2, three short bursts can be seen; the centre one is from a different motor unit and begins at a frequency greater than 200/sec. The two panels of A 3 show the record of another unit which began at a rate of about 250/sec., and gradually decreased over several seconds

to the slow irregular rate shown. This slow rate is broken by bursts from the same unit of two or three impulses where the initial rate is sometimes about 200/sec. Part B1 is from the innervated internal rectus of a high-section decerebrate cat. This burst of activity began spontaneously near 250/sec. (as heard on the loud-speaker) and continued until about 15 sec. later when at a rate of 110/sec. it abruptly ceased. This activity is believed to be peripheral in

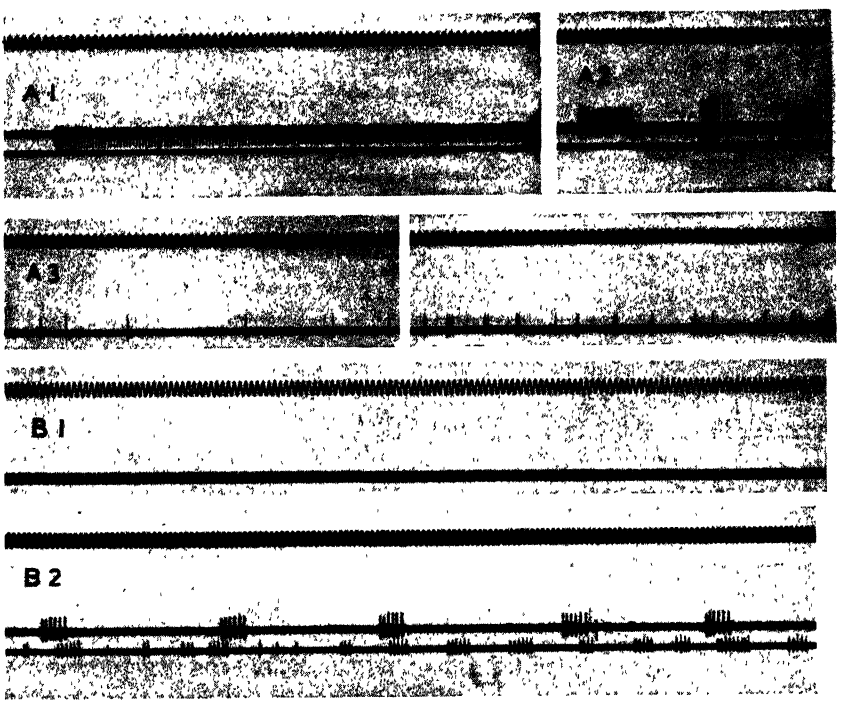

Fig. 1. Activity of peripheral origin picked up by wire electrodes. In parts A1, A2 and A3, they were inserted into the superior rectus muscle of a decerebrate cat with the third cranial nerve divided. In part B1, electrodes inserted into internal rectus of a cat whose brain stem was sectioned in front of the colliculi. In part B2, electrodes inserted into both internal and external recti of the same eye. Details in text. Time 50 c.p.s.

origin because of its resemblance to that obtained in the muscle isolated from the central nervous system, and because it could not be inhibited by appropriate movement of the head. Part B2 shows the activity of both the external and internal recti recorded in the upper and lower tracings respectively. The activity in each muscle is independent one of the other. In each muscle this discharge resulted from slight disturbance of the wire electrodes and continued for about 30 sec.

In general, the records of this non-reflex activity represented single units, and when there were more than one they stood out clearly from one another as

in Fig. 1, A2. This gives further support to the view that their origin is determined by the presence of the electrodes, for it is not at all unlikely that the electrodes may injure only one, two or three units within the whole field from which they may pick up impulses. On the other hand, when the reflex activity now to be described was picked up with wire electrodes records of single unit activity were rare, especially during vigorous stimulation, so that it was only with patience and good fortune that they were obtained.

Reflex activity

Muscle electrodes. The muscles were stimulated by head movements. For example, lateral flexion of the head and neck stimulated or inhibited the appropriate external and internal recti. Thus lateral flexion of the head to the right caused a burst of impulses in the left external and right internal recti. Returning it to the mid-line or flexing it to the left inhibited these muscles and stimulated the corresponding antagonists. Rotatory movements of the head were found suitable for stimulating the oblique muscles. The movements are designated clockwise or anti-clockwise as seen from in front of the animal, and are relative both to the horizontal plane and to the cat's body which lay prone and was not moved. Thus, an anti-clockwise twist of the head caused a burst of impulses in the left inferior oblique.

Fig. 2, part 1, shows the effect of laterally flexing the head and neck sharply to the right and maintaining flexion throughout the record. The action potentials are recorded from wire electrodes in the left external rectus. There is at first an asynchronous outburst of potentials from many units. After the initial outburst has subsided the action potentials from two units can be distinguished; about 1·1 sec. from the first spike one of these ceases to fire and thereafter the other continues firing, decreasing in frequency until some 4·5 sec. later (see second part of the panel), when it is of the order of 5–10/sec. While continuing at this rate it was terminated by flexing the head to the left. Fig. 2, part 2, shows a similar record from another portion of the same muscle. For the first five spikes the record is of a single unit, the intervals gradually decreasing until they correspond to a frequency of 75/sec. Thereafter the record becomes for a time multifibre and asynchronous, and later continues as a single fibre record until it is terminated by flexing the head to the left. Another record of this same unit was allowed to run for 15 sec. during maintained flexion. At 0·2 sec. after commencement the frequency was 65/sec., after 2 sec. it was 40/sec., after 8 sec. 35/sec., and during the fifteenth second before stopping the discharge by flexing to the left it was 38/sec.

These records are typical of many made with wire electrodes in the external rectus. During sharp flexion the records are multifibre and only rarely can single units be distinguished. During sustained flexion rates varying from 5 to 75/sec. were common. In some cases, as in Fig. 2, part 1, the rate fell over a few

seconds from 25 to between 5 and 10/sec. Other units, like the one in Fig. 2, part 2, fell in a few seconds from 75 to between 30 and 40 and remained there with little change for several seconds. In some records we have had single units discharging at slow rates for periods of several minutes. For example, one fibre in the internal rectus continued to discharge for at least 15 min., during which time its rate fell from 25 to 14/sec. In one experiment during the height of a discharge grouping of impulses occurred. This is illustrated in part 3 of

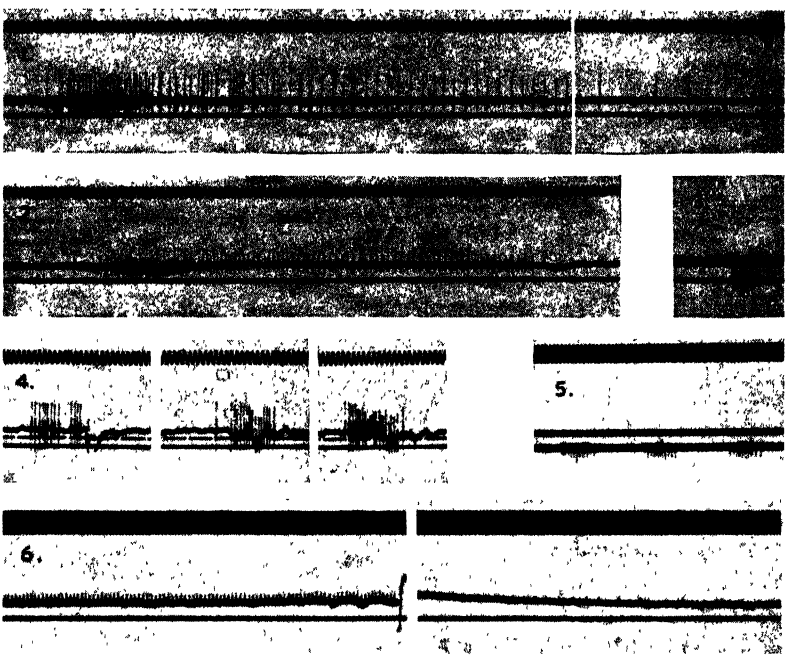

Fig. 2. Parts 1, 2 and 3 show records of activity in left external rectus obtained during head movements. In part 1, head was flexed to right and maintained there throughout record. Interval between panels is 3·7 sec. In part 2, activity was terminated by flexing head to left. In part 4, activity picked up by wire electrodes in left inferior oblique during anti-clockwise twists of head. In part 5, activity picked up from single fibre in nerve to left inferior oblique during anti-clockwise twists. In part 6, activity in single nerve fibre of nerve to left inferior oblique during galvanic stimulation of a labyrinth. At end of first panel is an artefact due to opening the circuit. Interval between panels 0·9 sec. Time 50 c.p.s.

Fig. 2. This record is of the external rectus during a sharp lateral flexion of the head; the maximum rate corresponds to about 65/sec.

Part 4 of Fig. 2 is typical of records obtained with a wire electrode in the interior oblique. Rotatory twists produced an outburst of action potentials which were not sustained while the new position of the head was maintained. In two cats it was possible to recognize a single unit throughout or in the greater portion of some of the records. Usually the centre of the record is

broken by an asynchronous multifibre discharge. Thus, Fig. 3 (open circles) illustrates an increase in frequency up to 166/sec. during a head twist. During the gaps in this graph the record showed a discharge of several units. Intervals as short as 5·6 msec. were commonly recorded; this corresponds to a frequency of 178/sec.

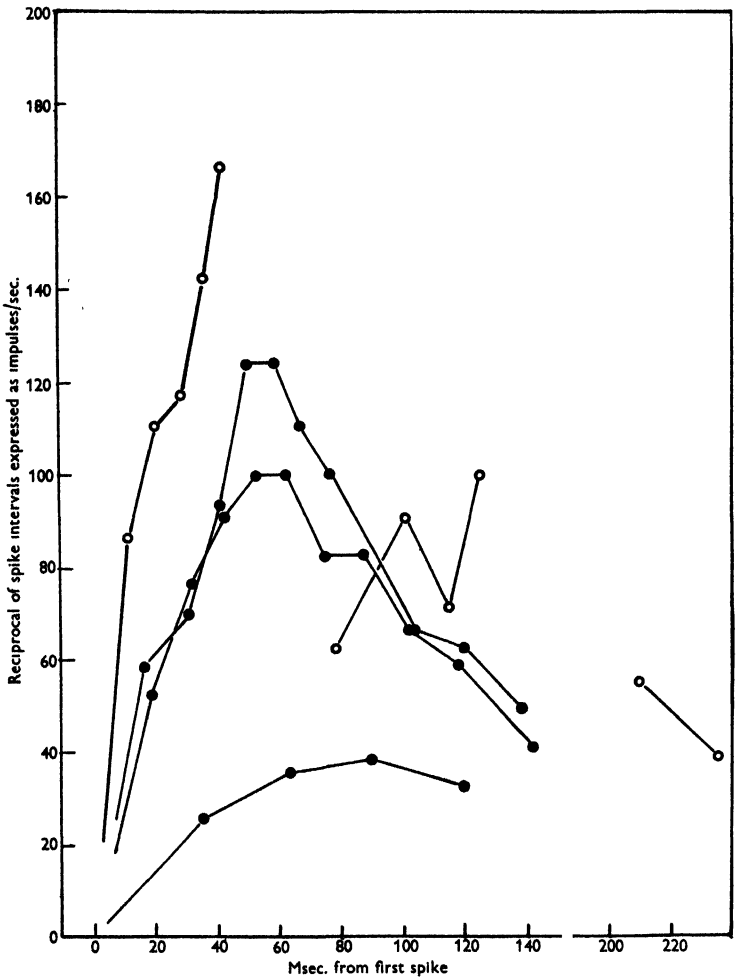

Fig. 3. Frequency of spikes picked up from single fibre of nerve to inferior oblique (dots), or from muscle electrodes (circles), during head twists. Ordinate: reciprocal of spike intervals expressed as impulses/sec. Abscissa: m.sec. from first spike.

Records obtained by wire electrodes in the external rectus of the goat during spontaneous movement of the eyes showed asynchronous activity in which it was not possible to count the rhythm of individual units. As the spontaneous movement subsided, units could be counted firing at rates varying from 5 to 75/sec.

Single nerve fibre preparations. Single fibre preparations of the nerve to the inferior oblique were made in two cats. Altogether five fibres were observed. There was no resting activity. On rotating the head, bursts of activity appeared similar to those recorded in part 5 of Fig. 2. Rotating the head as rapidly as possible by a movement of supination of the observer's right arm caused an outburst of some 10 or 12 impulses with intervals as short as 7 msec., corresponding to 144 impulses/sec. Weaker twists gave fewer impulses (3 or 4) at a lower rate as, for example, in lower curve, Fig. 3, in which three records of the one fibre are shown. During some twists instead of the simple rise and fall

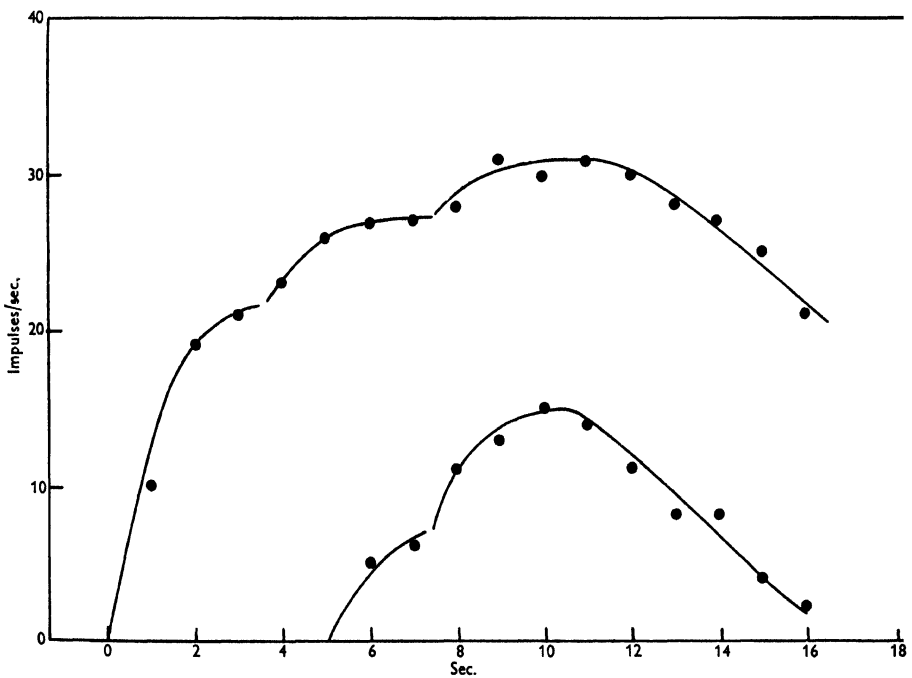

Fig. 4. Frequency of spikes, in preparation of nerve to inferior oblique during increasing and decreasing galvanic stimulation. For details see text. Between 5 and 6 sec. after stimulation was begun, responses from a second fibre appeared whose frequency/time response is shown in lower curve. Ordinate: impulses/sec. Abscissa: sec.

shown in these curves the frequency rose, for example, to 130, fell to 80, and rose again to 100/sec. before the final decrease.

Galvanic stimulation of the labyrinth caused a steady discharge, usually between 20 and 40/sec. but sometimes as great as 60/sec. More intense stimulation than this caused considerable movement of the whole animal. It was necessary to increase the intensity gradually or step by step by altering the resistance, and as a result the increase in frequency takes place in stages as indicated in Fig. 4. On the other hand, it was possible abruptly to open the

current and after the artefact so produced (Fig. 2, part 4) the frequency decreased almost linearly over 2 or 3 sec. The decay in Fig. 4 is more gradual than this because the stimulus was gradually decreased.

Fig. 4 also indicates how, as the intensity increases, a second fibre may be recruited. The record which began as a single unit showed, during the sixth second, impulses from another fibre. As the stimulus increased the first fibre attained a frequency of 31/sec., the second a frequency of 15/sec. As the stimulus decreased the second fibre ceased discharging when the first fibre had fallen to about the same level of activity as it showed when its companion had first appeared.

DISCUSSION

The results show that as the intensity of stimulation increases the extraocular muscles respond both with an increase in the frequency of individual units and a recruitment of new ones. In this respect they behave like the other muscles which have been studied. The recruitment of new units has made it difficult to study the upper frequency response of single units when wire electrodes are inserted into the muscles for, just as Adrian & Bronk (1929) found, the rhythm of the single unit is swamped by that of others during strong contractions. If this be so when the motor unit comprises many fibres, it is not surprising it makes observation difficult when the unit, as in the extraocular muscles, contains few. It is likely, therefore, that there are situations in which faster rates exist than are recorded here. Nevertheless, the maximum rates which it has been possible to record are apparently (with the possible exception of the 'double response' of Hoff & Grant (1944) higher than have ever been reported for other muscles. Thus Adrian & Bronk (1929) found, for single fibres to various muscles of the cat, that the highest frequencies were, for peronaeus longus 30, tibialis anterior 44, quadriceps 90, vastus lateralis 25, vastus medialis 65, and diaphragm 112/sec. Likewise, Denny-Brown (1929) found that the rate of discharge to the soleus varied between 5 and 20 during a stretch reflex and, when de-afferented, up to 35–40/sec. during a crossed extensor reflex. Also, in human muscles the discharge rates during voluntary contraction are relatively low, between 3 and 50/sec., but mainly near 20/sec. (Smith, 1934; Lindsley, 1935; Gilson & Mills 1941; Weddell *et al.* 1944). On the other hand, the rate of discharge to the extraocular muscles has been found commonly to reach during head twists the range of 120–175/sec.

Although such high rates were obtained it should be emphasized that relatively slow rates of discharge to the extraocular muscles were very common. Since the fusion frequency for tetanus is so high (Cooper & Eccles, 1930), it is clear that in order to have a smooth contraction in these circumstances the units must fire asynchronously, and during strong stimulation asynchronous discharges were in fact commonly found. As other workers have found, over

periods ranging from seconds to minutes, there was no evidence of rotation of activity from one unit to another; one unit continued discharging for at least 15 min. In one experiment synchronization of the impulses did occur at a rate of about 65/sec. These impulses were being picked up from only a small portion of the muscle. It is difficult to believe that this is a usual feature in the whole muscle if smooth contractions are to be obtained.

The patterns of discharge found in the muscle which is separated from the central nervous system resemble closely the patterns described by Adrian (1930) for injured mammalian nerve fibres isolated from the body. They appear to be due to the presence of the pick-up electrodes in the muscle. It is interesting that such discharges were so readily obtained from the extraocular muscles, whereas with other muscles they do not seem to have been described. One possible explanation may be the well-known fact that these muscles, in contrast to others, are strikingly rich in nerve fibres.

SUMMARY

Records were obtained from single fibre preparations of the nerve to inferior oblique and from muscle units by means of fine wire electrodes inserted into various extraocular muscles of the cat, 'decerebrated' above the colliculi.

Galvanic stimulation of a labyrinth caused rates of discharge up to 60/sec., but during sudden flexion or rotation of the head the rate reached over 170/sec. during movement and in the external rectus subsided to rates varying from 5 to 75/sec.

The presence of wire electrodes in the muscles readily provoked discharges, similar in pattern to those described by Adrian (1930) for isolated injured mammalian nerve. This is possibly attributable to the richness of the extraocular muscles in nerve fibres.

I gratefully acknowledge the help of Dr D. Whitteridge.

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A STUDY OF THE VIBRATIONS OF THE TYMPANIC MEMBRANE UNDER DIRECT VISION, WITH A NEW EXPLANATION OF THEIR PHYSICAL CHARACTERISTICS

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The specific nature of the vibrations of the tympanic membrane caused by sound waves impinging on the ear was first established by Politzer in 1861, by a series of experiments. After making preparations of the human middle ear by the removal of the tegmen tympani and of the inner labyrinthine wall, he attached fine glass threads, 10–12 cm. long, to the malleus and incus, and to each of these threads a fibre of a feather was fastened. The tones of organ pipes of different frequencies were conducted through the meatus to the membrana tympani. The vibrations of the ossicles were considerably magnified by these levers and could be made to register themselves on a blackened drum. These experiments were carried out with simple and combined tones. Regular wavy lines were traced with simple tones. The tones of two organ pipes which were an octave apart caused a large wave belonging to the deeper octave, on each of which appeared the small curve of the higher octave.

In Politzer's text-book (1909) experiments of another nature are also described. Starch granules were fixed to the ossicles and their vibrations were examined under a microscope having a micrometer. The white spot of the starch granules, when at rest, expanded into a line during the vibrations of the tympanic membrane and ossicles. The length of each line on the individual ossicles could be measured with the micrometer.

In the present series of experiments, dissections of the middle ear were made in which the tympanic membrane, malleus and incus were exposed from the inside (Pl. 1). A continuous sound was allowed to fall on to the tympanic membrane via the external auditory meatus, and the vibrations of the drum membrane and ossicles were examined under the illumination of a 'strobotac'. This has the effect of slowing down the visible vibrations in such a manner that they become discernible, and their character can be investigated. We believe

that this is the first time since the pioneer work of Mach & Kessel (1872, 1874) that the stroboscope principle has been applied to the study of sound transmission across the tympanum.

This direct visual inspection of the slowed-down drum vibrations was undertaken with two objects in view. First, we wished to confirm that the vibrations are specific in nature and have a direct relationship to the tone frequency, as had been shown previously by Politzer, using a different method. Secondly, we wished to investigate in detail the mechanism of the drum vibrations, and to determine to what extent the theoretical and mathematical calculations put forward by Helmholtz could be supported.

METHODS

Middle-ear preparations. In most of our experiments dissections of the cat's middle ear were employed. This has certain advantages, because the cat is used a good deal as an experimental animal and cats' heads are easily obtained. If the cat's head is placed in formalin solution immediately after death, very satisfactory middle ear dissections can be made when the part has been in the fixing solution for a week or so.

The cat's tympanic membrane is somewhat oval and has a maximum diameter of 8 mm.; the human tympanic membrane is more circular in shape and measures about 9 mm. across. Although the shape of the cat's malleus differs considerably from that of man, the length is approximately the same in both, i.e. about 9 mm.; and in each case a little more than half of the total length is embedded in the tympanic membrane.

The cat, in common with other carnivores, possesses a large tympanic bulla. The bone forming the floor of the tympanic bulla is thin, the bulla is easily broken into and the bone forming the floor of the tympanic cavity can be nibbled away. The cat's tympanic bulla is divided into two compartments, a larger postero-medial and a smaller antero-lateral. The thin partition between the two chambers must be removed before the tympanic membrane and the malleus become visible. The tympanic ring to which the drum membrane is attached must be carefully preserved. Most of the outer ear is removed, keeping only the innermost 1–2 cm. of the external auditory meatus. In the cat the meatus is cartilaginous to within 5 mm. of the tympanic ring. In such preparations the chain of ossicles is intact, the stapes remaining fixed in the oval window, and the tympanic membrane is seen somewhat on a slant.

Other middle-ear preparations were human ones, obtained from several dissecting-room subjects which had been in the anatomy department for over 12 months after the initial embalming by the injection of formalin solution into the femoral artery. The dissection is much more difficult, because there is no bulla, and careful chiselling and drilling of the bone around the tympanic cavity is required. When the inner wall of the middle ear is sufficiently loose, the piece of bone containing the labyrinth can be broken away. The stapes usually tears off at its junction with the incus and comes away with the promontory, leaving an intact drum with malleus and incus in position.

The appearance of such a dissection is seen in Pl. 1. During the experiments with the sound vibrations the drum is seen directly from the inner aspect, even more satisfactorily than in the cat's middle-ear dissections. While the experiment is in progress a small sound transmitter (see below) remains fixed in the short piece of the external auditory meatus which was left in position; the special meatus fitting supplied with the deaf aid was found very suitable for this purpose.

Apparatus used in the experiments. A Western Electric type deaf aid transmitter (no. 724 A) was utilized. The source of sound was an audio-frequency oscillator at frequencies from 300 to 1000 cyc./sec. The response curve of the transmitter is such that frequencies of 300–3000 cyc./sec. have to be used. Other frequencies do not excite the transmitter sufficiently. If it is desired to use other frequencies a special moving coil transmitter could be constructed for low frequencies. At

high frequencies (above 3000) the movement would be much smaller than at lower frequencies, also the flashing speed of the 'strobotac' would not be sufficiently rapid. The makers state that vibrations above about 1600 cyc./sec. cannot readily be followed by it. Voltages up to 5 V. were supplied to the transmitter. Any audio-frequency oscillator capable of supplying the necessary power and of matching the load of the transmitter, could be employed for this purpose. In our experiments a type 200 B Hewlett Packard resistance-capacity oscillator was used, having a frequency range from 20 to 20,000 cyc./sec. and a maximum output of 1 watt.

The illumination was obtained from a General Radio type 631 B 'strobotac' which can be adjusted to illuminate with flashes from a special neon lamp at frequencies from 10 flashes/sec. to 240 flashes/sec. The use of the stroboscope and the 'strobotac' for studying vibrations is well known. By adjusting the frequency of flashes so that the frequency of vibrations is near an integral multiple of the flashing speed, the apparent speed of the movement is reduced so that it can be followed by eye easily. The General Radio Company issue a brochure entitled *Eyes for Industry* in which the use of the 'strobotac' is described in detail.

A small-type dissecting microscope with three sets of eye pieces was used, allowing magnifications of 3·5, 7 and 10·5. The × 7 magnification was found to be sufficient. The whole tympanic membrane is seen in the microscopic field. The vibrations of the drum membrane can also be seen quite readily with the help of an ordinary hand-lens.

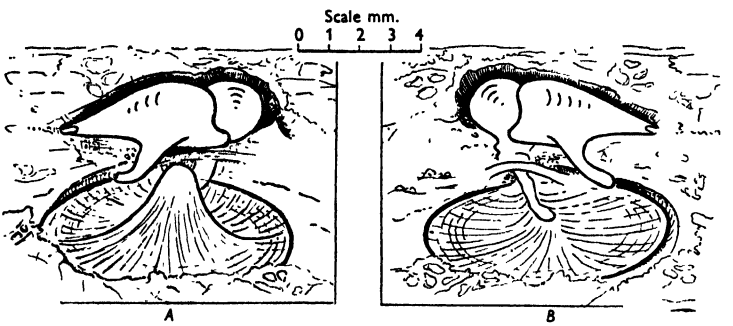
RESULTS

Description of the vibrations seen. The observations were made preferably in a dark room or at night, so that the only illumination of the drum membrane was by means of the strobotac, though later it was found that the vibrations were also seen easily in the daytime, provided some of the direct daylight was screened off from the part. The movements were made to appear slow by illuminating the tympanic membrane, malleus and incus with flashes at a frequency which differed from the oscillator frequency by 1 or 2 cyc./sec. Alternatively the frequencies of flashing and of the oscillator were adjusted so that the oscillator frequency differed by 1 or 2 cyc/sec. from an integral multiple of the flashing frequency. By adjusting the frequency of the oscillator or the frequency of the flashes near to this condition the apparent motion could be slowed down or arrested altogether, thus proving that the actual vibration was at the frequency supplied by the oscillator.

In these stroboscopic experiments it was clearly observed that the maximum vibration caused by the tone in the transmitter is at a point about halfway between the umbo and the periphery of the drum. It appears that the handle of the malleus represents a considerable 'load' which does not yield to the vibrations as easily as the other parts of the tympanic membrane which are not thus loaded. With tones of strong intensity the whole drum was seen to move in and out, causing a rocking movement of the malleus and incus around their axis of attachment.

Most of the observations were made on preparations of the cat's middle ear. We also observed the drum and ossicle vibrations under stroboscope illumination in an anaesthetized cat, in which the middle ear had been exposed in the way described above, after tying both internal carotids. Thus in the living

animal the drum and malleus movements were of the same nature as in the formalin-fixed dissection. Similarly, the same type of vibrations were seen extraordinarily well in the human middle-ear dissections when a continuous note was allowed to impringe on the tympanic membrane, although the ossicular chain was interrupted in these specimens (see Pl. 1). These stroboscopic observations have enabled us to put forward a modification of the Helmholtz theory (see Discussion and Appendix), but the work is being continued to determine the amplitudes of the drum and ossicle movements, as a function of sound intensity and of frequency.



Text-fig. 1. Drawings of the inner aspect of the tympanic membrane with the malleus and incus in position. *A* shows the normal condition of the cone (cf. Pl. 1). *B* shows the condition of the cone after decalcification of the surrounding bone; note the flattening-out of the cone.

It is necessary here to call attention to an important anatomical point in the shape of the drum. This membrane is drawn inwards towards the middle ear forming a cone with the summit at the umbo. At the same time it bulges towards the external auditory meatus in the portions extending from the umbo to the periphery (Pl. 1). As will be seen later, Helmholtz took full cognizance of this peculiar shape of the tympanic membrane in his theoretical calculations of the drum vibrations. He looked upon this special shape as particularly favourable for the transmission of sound waves.

In many text-books of histology the tympanic membrane is figured as much flatter than it is under normal conditions. This may be attributed to the effect of decalcification, as shown in Text-fig. 1. The action of the acid in decalcification probably destroys the effectiveness of the elastic fibres in the drum, the existence of which is described (Politzer, 1909; Maximow, 1930).

DISCUSSION

The observation that the maximum movement of the drum takes place about halfway between the umbo and the periphery confirms a theory first put forward by Helmholtz in 1862. He suggested that the drum itself is one of the main load-matching devices employed to increase the force acting on the stapes

This action has been compared with the function of a transformer which 'matches' say a low resistance loud-speaker to a high resistance amplifier valve (Beatty, 1932; Fletcher, 1929). In this case the stapes imparts its motion to the cochlear fluid which represents a high mechanical resistance. The air, not being a dense medium, represents a very low mechanical resistance. These same authors have considered the lever action of the ossicles and the larger area of the drum as compared with the smaller area of the oval window to be the main matching device concerned. The original theory of Helmholtz has been ignored by them. In analogy with many microphones and loud-speakers it has been assumed that the drum acts like a rigid cone or diaphragm mounted in a flexible support surrounding it. The tympanic ring is obviously rigid, but in these arguments it is assumed that a peripheral strip of the drum acts like the flexible support mentioned.

The Helmholtz theory of the action of the drum

In his book, *Tonempfindungen* (1862), Helmholtz describes the action of the tympanic membrane as follows. This description is so important and so relevant to the subsequent argument that it must be given in full.

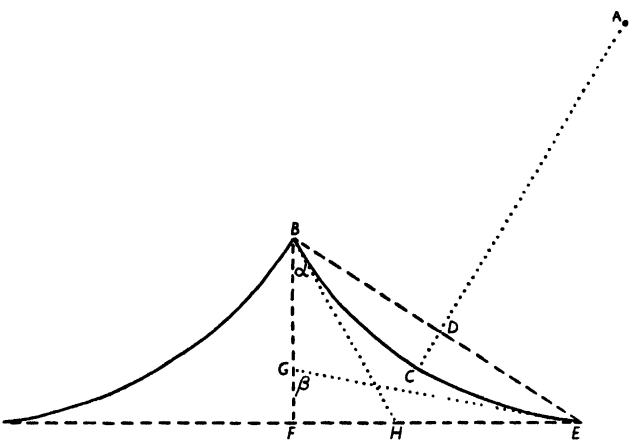
The chief means of reinforcement is due to the form of the drum membrane. It has already been mentioned that its middle or umbo is drawn inwards by the mandle, so as to present a funnel shape. But the meridian lines of this funnel drawn from the umbo to the circumference, are not straight lines; they are slightly convex on the outer side. A diminution of pressure in the auditory passage increases this convexity, and an augmentation diminishes it. Now the tension caused in an inextensible thread, having the form of a flat arch, by a force acting perpendicular to its convexity, is very considerable. It is well known that a sensible force must be exerted to stretch a long thin string into even a tolerably straight horizontal line. The force is indeed extremely greater than the weight of the string which pulls the string from the horizontal position. In the case of the tympanic membrane, it is not gravity which prevents its radial fibres from straightening themselves, but partly the pressure of the air, and partly the elastic pull of the circular fibres of the membrane. The latter tend to contract towards the axis of the funnel-shaped membrane, and hence produce the inflexion of the radial fibres towards this axis.

By means of the variable pressure of air during the resonant vibrations of the atmosphere this pull exerted by the circular fibres is alternately strengthened and weakened, and produces an effect on the point where the radial fibres are attached to the tip of the handle of the malleus, similar to that which would happen if we could alternately increase and diminish the weight of a string stretched horizontally, for this would produce a proportionate increase and decrease in the pull exerted by the hand which stretched it.

In a horizontally stretched string such as has been just described, it should be further remarked that an extremely small relaxation of the hand is followed by a considerable fall in the middle of the string. The relaxation of the hand, namely, takes place in the direction of the chord of the arc, and easy geometrical considerations show that chords of arcs of the same length and different, but always very small curvature, differ very slightly indeed from each other and from the lengths of the arcs itself. This is also the case with the drum membrane. An extremely little yielding in the handle of the malleus admits of a very considerable change in the curvature of the membrane. The consequence is that, in resonant vibrations, the parts of the tympanic membrane which lie between the inner attachment of this membrane to the malleus and its outer attachment to the tympanic ring, are able to follow the oscillations of the air to a considerable extent, while the motion of the air is

transmitted to the handle of the malleus with much diminished amplitude but much increased force. After this, as the motion passes from the handle of the malleus to the stapes, the leverage already mentioned causes a second and more moderate reduction of the amplitude of vibration, with corresponding increase of force.

The photographs of the drum in Pl. 1 illustrate Helmholtz's description, and Text-fig. 2 is a diagram which represents a horizontal section of the drum membrane through the umbo. The radial fibres of the membrana tympani are assumed to lie on the arcs of a circle (BCE) with its centre at A. Helmholtz gives a formula for the difference between the length of the chord and the length of the arc as $\frac{8}{3}\frac{s^2}{l}$, where l is the length of the arc (BCE) and s the distance (DC) of the centre of the chord from the arc. A more accurate formula is given in the mathematical appendix, but for the actual shape of the drum, the approximation of Helmholtz is sufficiently accurate.



Text-fig. 2. Diagram of a horizontal section of the tympanic membrane through the umbo. Radius $AE = AB = AC = r$. Arc $BCE = l$. Chord $BE = y$. Distance $DC = s$. Distance $FB = g$.

This treatment only correlates the movement of the drum halfway between the umbo and the periphery with the movement of the malleus. A more accurate treatment requires the pressure on the whole membrane to be considered. Helmholtz also gives a treatment of this problem. He calculates the force on the membrane as

$$\text{force} = \frac{\pi R^2 p \cos\alpha}{\cos\beta - \cos\alpha},$$

where R is the distance EF, α is the angle FBH, β is the angle FGE (see Text-fig. 2), and p is the pressure.

$\pi R^2 p$ is obviously the force on a diaphragm having the same area as the aperture of the tympanic ring. The expression $\frac{\cos\alpha}{\cos\beta - \cos\alpha}$ is therefore a measure of the gain due to the lever action of the tympanic membrane itself.

A modification of the Helmholtz theory of the movements of the membrana tympani, involving the action of the tensor tympani muscle

Helmholtz does not suggest that the tensor tympani has any specific function in the mechanism of the drum vibrations. We would like to put forward the theory that the pull of the tensor tympani is essential to the lever action of the drum as described by Helmholtz. If the tensor tympani is relaxed then this lever action could only take place provided the membrane were sufficiently stiff to resist any compression. We do not believe that the tympanic membrane is sufficiently stiff in a living animal to act in this manner. We have also observed under stroboscopic illumination that the membrane can move beyond the point where the radial fibres are straight. In other words, it can become slightly concave towards the external auditory meatus, if very loud sounds fall on the tympanic membrane. If the mechanism remained the same, then a considerable second harmonic component would be introduced into the motion of the malleus from this point onwards. Helmholtz was of the opinion that the membrane could not yield to such an extent without tearing.

We suggest that, when the tensor tympani is contracted the lever mechanism functions as described by Helmholtz; but when the tensor tympani is relaxed the drum acts less effectively on the malleus, because it does not take advantage of the lever action. In the relaxed condition of the tensor tympani only a small area of the tympanic membrane near its centre is effective, and the efficiency is considerably reduced. On the other hand, when the tensor tympani is contracted the matching mechanism is effective and the ear is specially sensitive. In the relaxed condition of the tensor tympani the matching device does not function and the ear is less sensitive. Therefore we believe that the tensor tympani comes into action with the smaller intensities of sound. It may be compared to the muscle fibres controlling the iris, which adjust the eye to conditions of illumination level.

Loudness contours. One consequence of this theory agrees very well with observations on the loudness contours. The contraction of the tensor tympani increases the stiffness of the drum and middle-ear mechanism. At low frequencies, we would therefore expect that the gain in sensitivity due to the lever action would be counterbalanced by a loss due to increased stiffness. This agrees with the shape of the equal loudness contours as observed by Fletcher & Munson (1933) and by Churcher & King (1937). In both cases the contours show that at low intensity levels the ear is relatively less sensitive than at greater intensity levels. For example, at a loudness level of 10 phons the intensity levels at 100 and 1000 cyc./sec. are 40 and 10 db. respectively above 0·0002 dyne/sq.cm. (Churcher & King, 1937). If these tones, which are judged to be equally loud, are both increased in intensity by 50 db. to a level of 90 and 60 db. respectively above 0·0002 dyne/sq.cm., the loudness levels are then

80 and 60 phons. The lower note is relatively much louder at the greater intensity.

Anatomically this theory also gives an explanation for the fact that the drum and the tensor tympani are presumably present in all mammals, whereas some mammals have minute ossicles which may almost be described as vestigial; for example, in many species of the Rodents and Insectivores (Keen & Grobbelaar, 1941). This is because the drum is essential for the lever action required, whereas the ossicles contribute a much smaller leverage to the motion.

Measurements of the curvature of the membrana tympani and calculation of the lever gain from this action

Pl. 1 A is a photograph of the tympanic membrane from a specimen obtained in the dissecting room. Pl. 1 B is the same specimen with a probe pressing against the malleus above the axis of rotation and thus stretching the drum. Measurements taken on these two photographs are summarized in Table 1. For purpose of the argument it will be assumed that the umbo is equidistant from the anterior and posterior margins of the tympanic membrane, and that the curve may be measured either forwards or backwards. It can be seen from the values of the mechanical advantage calculated from the curvature of the drum that the total gain is of the order of a factor of 4 when the drum is under tension, and of the order of 3 when the drum is not under tension. This difference is not sufficient to account for a sensitivity control of much consequence.

TABLE 1. Showing the measurements obtained from the photographs in Fig. 1, and the calculations of lever gain

	Length of chord (y)	Chord to centre of arc (s)	Ratio s/y	dy/ds (see Text-fig. 4)	g/y	Mechanical advantage $= \frac{y}{g} \times \frac{ds}{dy}$	Angle α	Angle β	$\frac{\cos\alpha}{\cos\beta - \cos\alpha}$
Pl. 1 A	4·5	0·49	0·11	0·60	0·54	3	31°	73°	1·5
Pl. 1 B	4·5	0·37	0·083	0·45	0·58	4	34°	70°	1·7

(The measurements are in mm. and for most of the symbols consult Text-fig. 3.)

If the lever action does not function when the tensor tympani is relaxed, as we postulate, then, in the one case we have a mechanical advantage of 4 (tensor tympani contracted), and in the other case no advantage, i.e. a factor of 1 (tensor tympani relaxed). This is not in conflict with the previous calculation which determined a factor of the order of 3 when the drum was not under tension. The calculations of mechanical advantage in Table 1 were made with the assumption that the drum lever action, described by Helmholtz, is always effective. We now hold that the drum lever action cannot be effective when the tensor tympani is relaxed. The mechanical advantage represented by a factor 4 would account for a sensitivity control having a range of about 12 db.

With the second method of calculating the advantage to be gained from the shape of the drum membrane, i.e. by the use of the formula $\frac{\cos\alpha}{\cos\beta - \cos\alpha}$, it would appear at first that the control which could be exercised by the tensor tympani is only small (about 5 db.). It must be borne in mind, however, that with the tensor tympani relaxed probably only a small part of the area of the drum is effective. From our observations we would suggest that only about half the radius or one-quarter of the area is effective. This would represent a ratio of about 7 between the two conditions or a sensitivity control of about 17 db.

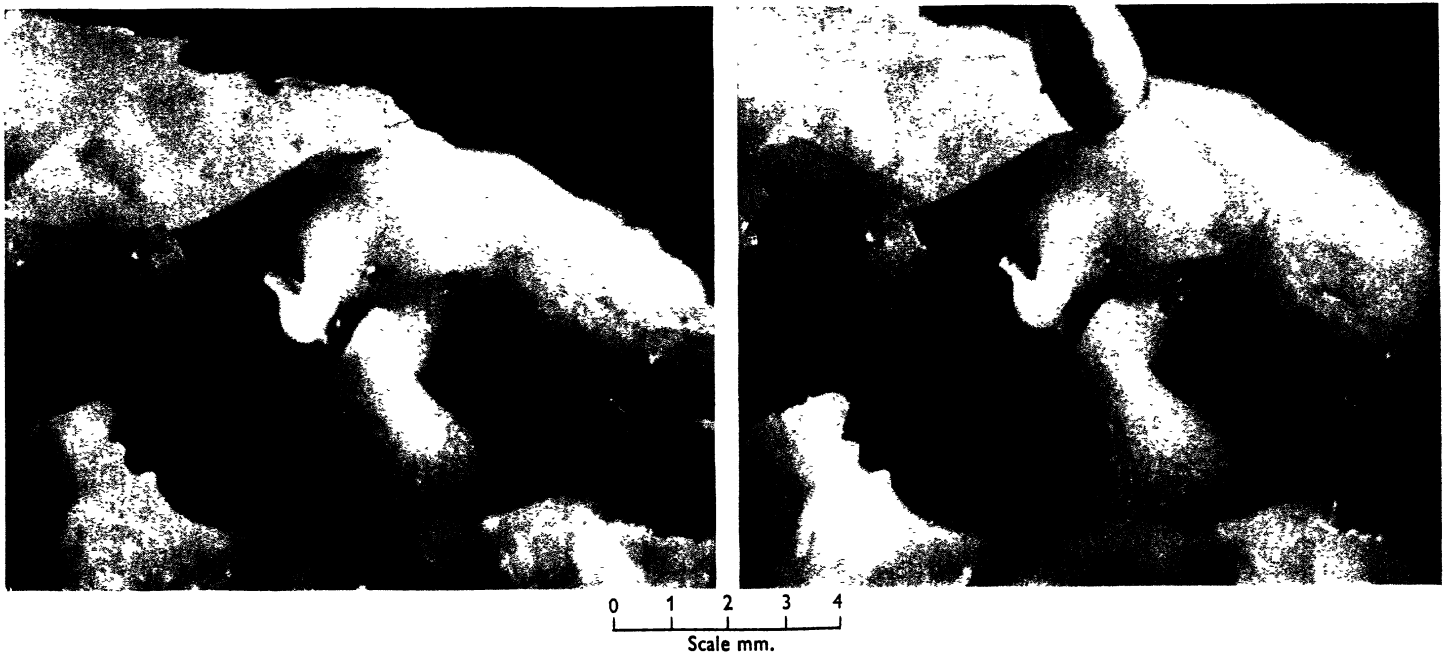
These measurements have been made on a drum fixed by formalin. The figures should therefore be regarded as giving only qualitative results, which enable us to calculate the order of magnitude of the quantities concerned. It is quite possible that the living drum is much more elastic and that it can be tensioned beyond the point shown in Pl. 1B. This would give much more control to the tensor tympani. Thus, if in the living drum it were possible for the ratio s/y to reach a value of say 0·04, then the mechanical advantage would be about 8, representing a range of control of 18 db.

SUMMARY

The vibrations of the membrana tympani and of the ossicles which arise when a continuous tone is sounding in the external auditory meatus, have been studied under stroboscopic illumination and with slight magnification of the middle-ear structures. These observations on the cat's middle ear and on human specimens show that the drum vibrates with the greatest amplitude approximately halfway between the umbo and the periphery. This confirms the theory of the action of the drum membrane originally put forward by Helmholtz.

In the authors' opinion the theory of Helmholtz does not account for the drum movements at large amplitudes. They therefore suggest a modification of the Helmholtz explanation of the mechanism of the drum movements, which involves the action of the tensor tympani muscle. The tensor tympani is considered to have the function of adjusting the effectiveness of the membrana tympani in sound transmission.

We wish to thank Dr N. Sapeika of the Physiology and Pharmacology Department for his kind assistance with the stroboscopic observations in the living animal. One of us (J.A.K.) gratefully acknowledges the help received from a Research Grant of the University of Cape Town.



Photographs of the inner aspect of the human tympanic membrane with the malleus and incus in position, after removal of the inner labyrinthine wall: seen from below. Photographs A and B were taken with the specimen in the same position. In B, however, some pressure was exerted with a probe on the malleus in the epitympanic recess, causing a little inward swing of the handle of the malleus with a slight increase of the cone. The increase of the cone amounted to about 0·3 mm.

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MATHEMATICAL APPENDIX

The following assumptions are made:

(1) The action of the tensor tympani subjects the radial fibres of the drum to a constant tension. In Test-fig. 2, which represents a diagrammatic section of the drum through the umbo, the arc l remains constant in length because the tension is constant.

(2) These radial fibres lie on the arc of a circle (centre at A).

It is now desired to calculate a small variation of the distance y as a function of a small variation of the distance s for different values of s/y, the length l being constant.

If r is the radius of the circle, then

$$s(2r-s)=(\tfrac{1}{2}y)^2. \tag{1}$$

If θ is the angle subtended by l, then

$$\theta=\frac{l}{r}. \tag{2}$$

From (1)

$$r=\frac{s^2+(\frac{1}{2}y)^2}{2s},$$

$$\sin\frac{\theta}{2}=\frac{sy}{s^2+(\frac{1}{2}y)^2}=\sin\frac{sl}{s^2+(\frac{1}{2}y)^2},$$

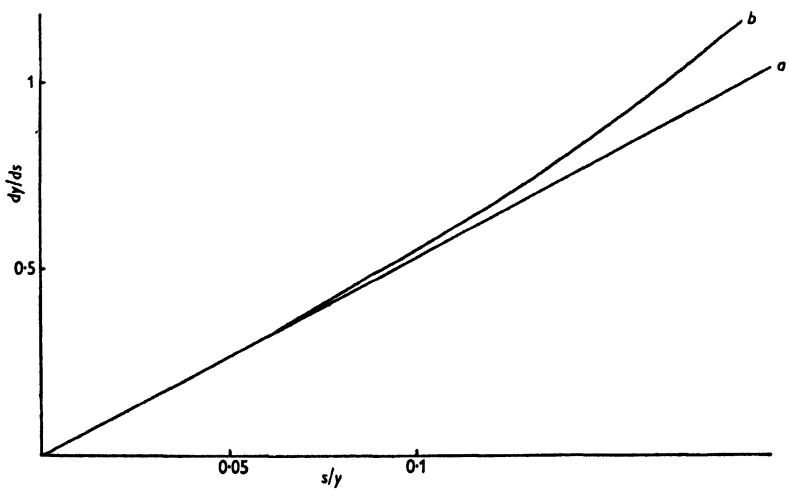
or

$$\sin\frac{sl}{s^2+(\frac{1}{2}y)^2}-\frac{sy}{s^2+(\frac{1}{2}y)^2}=0=u,$$

differentiating u partially by y and s and using the relationship

$$\frac{dy}{ds}=-\frac{\partial u}{\partial s}\Big/\frac{\partial u}{\partial y},$$

and simplifying
$$\frac{dy}{ds}=\frac{y-l\cos\dfrac{ls}{s^2+(\frac{1}{2}y)^2}}{s-\dfrac{lsy}{(\frac{1}{2}y)^2-s^2}\cos\dfrac{ls}{s^2+(\frac{1}{2}y)^2}}.$$



Text-fig. 3. Curves illustrating the lever action of the tympanic membrane. The abscissa represents the distance of the chord from the arc divided by the length of the chord (see Text-fig. 2). The ordinate is the differential quotient dy/ds. a = calculation according to Helmholtz; b = calculation according to mathematical appendix.

This curve is plotted in Text-fig. 3. The movement of the 'malleus' for a small movement of a point midway between the malleus and the periphery is therefore $\frac{dy}{ds}\times\frac{g}{y}$. The measurements obtained from the photographs in Fig. 1 are summarized in Table 1.

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A QUANTITATIVE METHOD FOR THE STUDY OF THE REACTIONS OF THE ISOLATED CAT'S IRIS

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For many years, the action of a drug upon the iris was observed qualitatively after instillation of that drug into the conjunctival sac of the intact eye. A more direct approach to the problem was made by Dale & Laidlaw (1912), who studied the action of certain compounds upon a cat's iris isolated in a dish of Ringer solution. Other workers have examined the reactions of isolated strips of the dilator or sphincter muscles of the iris (e.g. Joseph, 1917, 1921, and Poos, 1926, 1928). This paper deals with a photoelectrical method of measuring the changes in pupil area, which permits quantitative studies of the action of a drug on the cat's iris isolated in a 'perspex' chamber through which oxygenated Ringer-Locke fluid flows.

METHOD

The cats were anaesthetized with ether followed by chloralose. The eyes were enucleated and dissected as rapidly as possible in cold Ringer-Locke fluid. The cornea was removed anterior to the corneosclerotic junction. The optic nerve head was excised from the globe, and six radially running incisions were made from this area to points just short of the ora serrata. The six flaps of sclera so formed were gently scraped to remove the adherent retina and were subsequently used to suspend the iris in the chamber. The lens, its capsule, and any remaining vitreous humour were next removed. The 'perspex' chamber, in which the iris was mounted, was made in two halves. Six gramophone needles, projecting ⅛ in. from one half, transfixed the six scleral flaps and then engaged in six holes drilled in the other half of the chamber. The two halves of the chamber could be bolted together and rendered watertight by an interposed rubber washer (Fig. 1). It was important that there should be no accumulation of bubbles in the chamber to interfere with the optical characteristics of the apparatus and that the iris should be adequately supplied with Ringer-Locke fluid fully saturated with oxygen at 37° C. This was achieved by oxygenating the Ringer-Locke stock solution in a large aspirator in a water-bath maintained at 37° C. by a gas thermoregulator. The Ringer-Locke fluid, thus warmed and oxygenated, was conducted to a pyrex glass spiral immersed in a second and smaller water-bath close to the preparation in which the final adjustment of its temperature was made. As a precaution, the T piece containing the thermometer recording the temperature of the fluid immediately before it entered the chamber acted as a final bubble trap. All tubing conveying the warmed Ringer-Locke solution was insulated by asbestos lagging.

A constant rate of flow of fluid was maintained through the cell, adjustment being controlled by tap C in the tube leading from the stock aspirator and screw clip C' (fine control) on the outflow

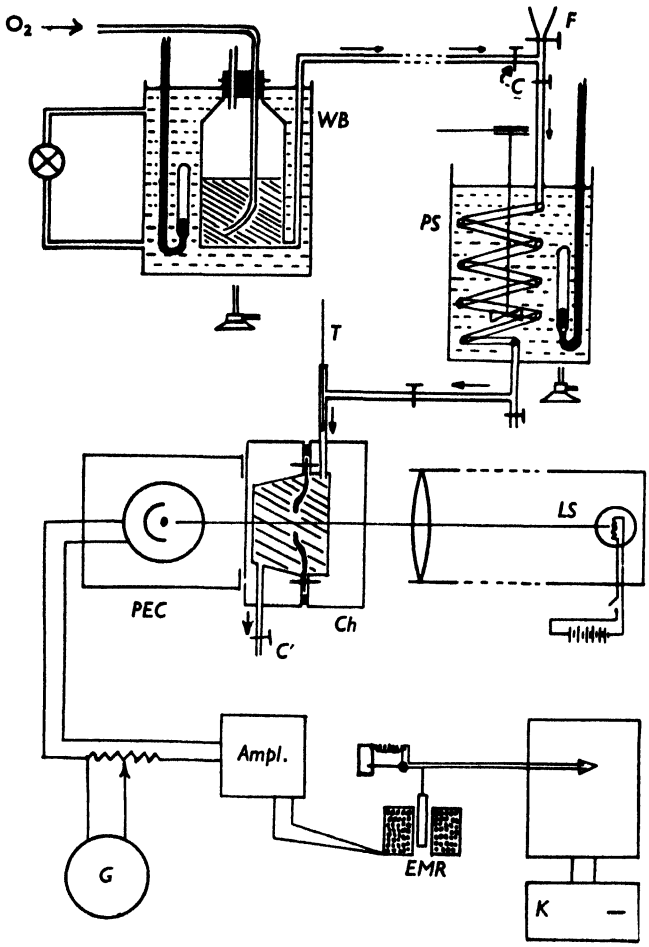

Fig. 1. Diagram of apparatus used in recording reactions of the isolated iris. The stock solution of Ringer-Locke fluid is warmed to 37° C. and oxygenated in large aspirator in water-bath *WB*. The fluid is conducted by lagged tubing, via tap *C*, which is the coarse control of flow, to a pyrex-heating spiral in a small gas thermo-regulated water-bath, *PS*, where the final regulation of temperature is made near the preparation. The fluid then passes, via a T piece which holds a thermometer, *T*, to the perspex chamber, *Ch*, which contains the iris. The constant light source, *LS*, is shown in the collimator and the light transmitted by the pupil falls upon the photoelectric cell, *PEC*. The current generated is led to the amplifier, *Ampl*, and part is used to deflect the galvanometer *G*. The output of the amplifier operates an electromagnetic recorder, *EMR*, which writes directly on a kymograph (*K*). C' is a tap used as a fine control of flow in the outlet tube.

tube from the chamber. The outlet of the outflow tube was arranged to be slightly above the level of the fluid in the chamber. With these precautions it was possible to maintain the iris in a state in

which it would react readily to drugs over a period of 6–10 hr. The chamber remained free from air bubbles and permitted continuous recordings to be made.

The optical part of the apparatus consisted of a constant source of light obtained from an artificially 'aged' 36 W. motor-car headlamp bulb connected to a large capacity accumulator. The light from this lamp was rendered parallel by a collimator and thrown on to the upper surface of the chamber and thus on to the anterior surface of the iris. The surfaces of the chamber through which this beam of light passed were parallel to one another, perpendicular to the beam and were carefully polished. The light transmitted by the pupil, dependent upon its area, fell upon an antimony screen photoelectric cell in a light-tight box. The output from the photoelectric cell was led to a d.c. amplifier and a portion of it was fed by an Aerton shunt to a quick-acting galvanometer. The output of the d.c. amplifier operated an electro-magnetic recorder writing directly on a kymograph. The recorder consisted of a hollow core coil former upon which approximately 8 oz. of 40 s.w.g. enamelled copper wire were wound. This coil was connected in series with the cathode

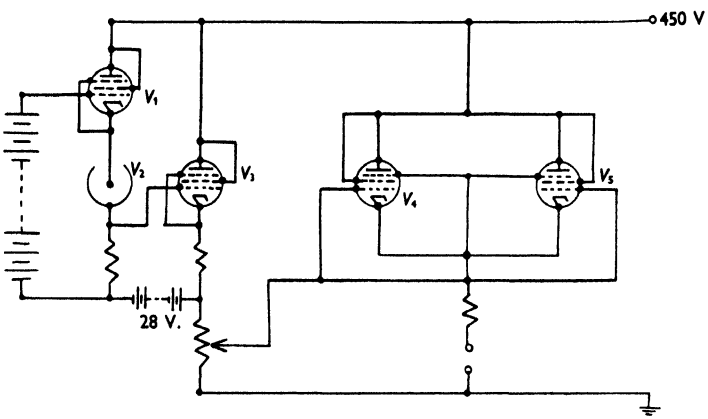

Fig. 2. Circuit of the d.c. amplifier with an overall current gain of 10,000. V_1, V_4 and V_5 are of type SP41 (VR 65), V_2 is an antimony screen photoelectric cell (RCA929), and V_3 is of type EF36 (VR56). The electromagnetic recorder is connected in series with the cathode load resistance of V_4 and V_5 to the two terminals indicated. A 100 V. decade operates the photoelectric cell.

resistance of the output valves of the amplifier. The current flowing in the coil exerted an electromagnetic effect upon a movable soft iron rod attached to the writing lever and suspended in the hollow core of the coil. Such a recorder responded to changes in the current flowing in the coil in a somewhat unpredictable manner owing to the residual magnetism that developed in the soft iron rod. This hysteretic effect was completely overcome by applying a small a.c. field by another winding on the coil. Under these conditions the excursions of the soft iron rod for any given currents in the main winding of the coil were reliable and repeatable. The perspex chamber was mounted upon a turn-table so that either the chamber or a perforated disk could be interposed in the beam of light falling upon the photoelectric cell. The perforations in the disk corresponded to a range of pupil areas between full constriction and wide dilatation and were accurately made for use in checking the response of the apparatus to varying amounts of light falling upon the photoelectric cell.

I am indebted to Mr V. I. Little of the Physics Department of King's College, London, who designed the circuit for the amplifier (Fig. 2) and the electromagnetic recorder. The d.c. cathode-coupled amplifier had an overall current gain of 10,000 times. The circuit is neither sensitive to variations in h.t. nor to variations in valve characteristics. V_1 serves as a battery isolater and enables a constant voltage to be maintained across V_2, the photoelectric cell, and its load. (V_1 may

be dispensed with if a small drain on the battery can be tolerated.) V_3 is a cathode-coupled amplifier giving a gain of 100 times and produces an output that is in phase with the input. V_4 and V_5 constitute a normal 'cathode-follower' current amplifier.

The drug whose action upon the iris was to be studied, was made up in 30 ml. of oxygenated Ringer-Locke fluid obtained from the stock aspirator and the resulting solution poured into funnel *F* (Fig. 1). At the required moment the solution could be introduced into the pyrex-heating spiral from which it passed to the iris.

Thus, it is possible to observe the changes in pupil area as an indication of the action of the drug upon the iris musculature by following the galvanometer's deflexions and/or observing a tracing on a kymograph by the direct-writing electromagnetic recorder.

Characteristics of the apparatus. The galvanometer deflexion when each of the perforations of known area on the test disk was interposed in the light beam was plotted against the area of that perforation. These points fall along a straight line. The relation between the microamperes produced by the photoelectric cell and the deflexions of the galvanometer using the perforated test disk, was also linear. When the photoelectric cell was exposed for 1 hr. continuously to the amount of light passed by a small- or moderate-size perforation in the disk there was no falling off in the amount of current generated by the photocell. With the perforation corresponding to full dilatation of the pupil, there was no falling off in galvanometer deflexion over a 30 min. period, but after this there was some evidence of 'fatigue' of the photoelectric cell. In practice, the photoelectric cell was never subjected to such fatigue for the pupil was never allowed to remain fully dilated for longer than a few minutes at a time.

The amplifier characteristics were such that there was no detectable falling off of the current in the output when a constant illumination of the type encountered experimentally fell upon the photoelectric cell.

It was found that the quick-acting galvanometer gave a very accurate picture of the events following the addition of a drug to the system, while the direct-writing electromagnetic recorder was particularly useful to give warning of these changes and to provide an immediate recording of events so that the succeeding steps of each experiment might be planned to suit each preparation. The recorder was found to respond reliably to the rates of change of pupil size encountered.

It was concluded that the optical and electronic characteristics of the apparatus could be relied upon to give accurate and continuous information regarding the area of the pupil under observation.

RESULTS

The pupil constricted markedly in the manner described by Dale & Laidlaw (1912) shortly after it was mounted in the perspex chamber. After oxygenated Ringer-Locke fluid had flowed through the chamber for 10–30 min., the pupil usually dilated to a degree adequate for the present studies. If it did not, a single small dose of adrenaline produced a prompt dilatation followed by constriction rather less than was present immediately before the adrenaline was given. Such a reaction of the iris was only seen following the initial dose of adrenaline to a fresh preparation. Normally, the pupil constricted to its original size following adrenaline (Fig. 6). It should be noted, however, that the reactions of a fresh iris to the first and second doses of the first drug were often unreliable and recordings were not made until this phase had passed. With the illuminations used in these experiments no evidence of any direct reaction of the iris to light was observed. In none of the fifty irides studied was there any spontaneous rhythmical activity of the type reported by Heath & Geiter (1939) in isolated strips of iris muscle.

The action of acetylcholine

The miotic effects of a series of progressively increasing doses of acetylcholine are illustrated in Fig. 3 in which the galvanometer deflexions following 1, 10, 100 and 1000 μg./ml. of acetylcholine are plotted against time. In Fig. 4, a group of responses is plotted for a series of doses of acetylcholine progressively reduced on a logarithmic basis. It will be seen that as low a concentration as 0·001 μg./ml. of acetylcholine (total dose being 0·03 μg.) produced a discernible constriction. The responses increased in size as the dose was made larger. The time for maximal constriction of the iris following a dose of acetylcholine was

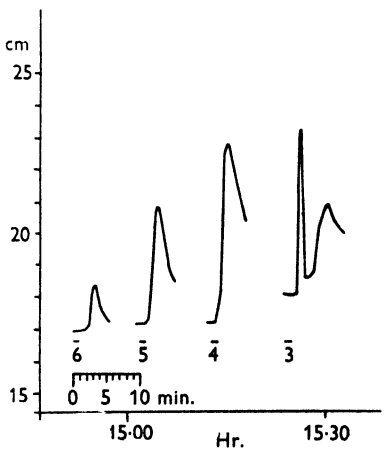

Fig. 3. The action of doses of acetylcholine, increasing progressively on a logarithmical basis, upon the isolated iris. The curves are constructed from readings of the deflexion of the galvanometer taken every 15 sec. plotted against time (constriction upwards). The absolute time is shown on the abcissa and a scale indicates minutes. The figures 6, 5, 4 and 3 refer to 30 ml. doses of the acetylcholine dilutions containing respectively 1, 10, 100 and 1000 μg./ml. applied to the iris during the period indicated by the short bar. The last named dose caused the 'dual response' consisting of a rapid constriction and dilatation of the isolated iris followed by a smaller and slower constriction. Note the rapid attainment of each constriction, the time for which is practically independent of the dose of acetylcholine applied.

approximately 2 min. and varied but little whether the dose was large or small. The time of the dilatation phase was longer and, as the dosage was increased, it became more protracted. As a control procedure, 30 ml. of oxygenated Ringer-Locke fluid was withdrawn from the stock aspirator and applied to the iris, via funnel *F*, at intervals during the experiments. These applications produced no alterations in pupil size. When the percentage of maximum action obtained with each of the doses of acetylcholine in any one isolated iris as plotted against the logarithm of the concentration of the respective doses, the resulting graph was of the form shown in Fig. 5*a*, *b*.

The reaction of the isolated iris to 1000 μg./ml. or more of acetylcholine deserved special note on account of its dual nature (Fig. 3). The first phase was an extremely rapid and profound constriction which gave place to an equally

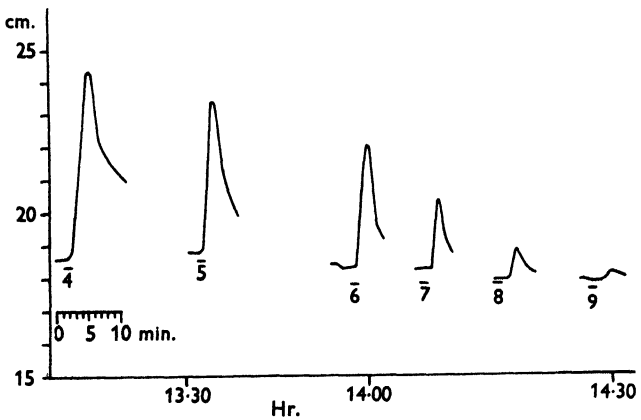

Fig. 4. Curves illustrating the action of doses of acetylcholine, decreasing progressively on a logarithmical basis, upon the isolated iris. The figures 4, 5, 6, 7, 8 and 9 refer to 30 ml. doses of acetylcholine containing 100, 10, 1, 0·1, 0·01 and 0·001 μg./ml. respectively. The presentation of the curves is similar to that in Fig. 3.

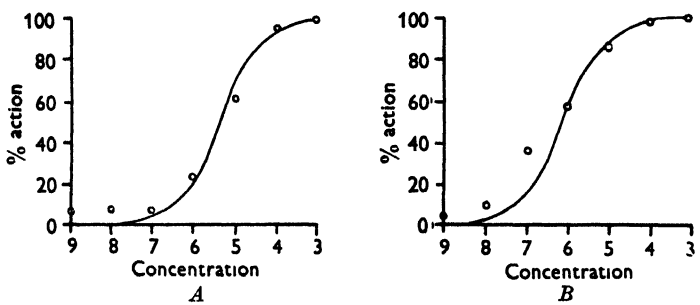

Fig. 5. Concentration-action curves of the isolated iris with acetylcholine. Ordinate: percentage of maximum action resulting from the dose of the drug tested. Abscissa: negative logarithm of the concentration of the drug used. The curves drawn are those described by the theoretical equation: $Kx = \frac{y}{100 - y}$. *A* and *B* are derived from Figs. 3 and 4 and illustrate the concentration action curves in the case of acetylcholine.

rapid dilatation of the pupil approximately to its original size, both these reactions being complete within about 2 min. Immediately following, there was a second but slower and less profound pupillary constriction, maximal in 3 min. after which there was a slow dilatation of the pupil to its original size (Fig. 3). This dual reaction could be repeated several times in each iris and was observed in every instance in which 1000 μg./ml. or more of acetylcholine was applied to an isolated iris.

The acetylcholine concentrations used in these experiments were made by diluting samples of a stock solution of acetylcholine in absolute alcohol (1 g./100 ml.) with warm oxygenated Ringer-Locke fluid from the stock aspirator. It was necessary to exclude the possibility that the alcohol in the dilutions used exerted an effect upon the isolated iris musculature, particularly in respect to the dual reaction described above. When 3 ml. of absolute alcohol were diluted to 30 ml. with Ringer-Locke fluid and applied to the iris, no detectable changes in pupil area were observed. Lower concentrations of alcohol were also inactive. It was concluded that the alcohol present in the dilutions of acetylcholine used had no action upon the isolated iris in these studies.

Occasionally acetylcholine produced an anomalous pupillary response, namely, dilatation, but such reactions were very rare and could not be repeated immediately after one of these unexpected events had occurred. No procedure was found which could be relied upon to produce these unusual reactions of the isolated iris to acetylcholine.

The action of atropine

Atropine sulphate alone had no action upon the isolated iris but in small dosage (1·0 – 10 μg./ml.) it markedly reduced the sensitivity of the preparation to acetylcholine. There was no differential action of these concentrations of atropine upon either phase of the dual reaction of the isolated iris to 1000 μg./ml. or more of acetylcholine. With 100 μg./ml. of atropine sulphate, the sensitivity of the iris to all concentrations of acetylcholine was abolished, and no recovery was seen even after prolonged passage of Ringer-Locke fluid through the cell.

The action of anticholinesterases

In a few experiments eserine (0·1–1·0 μg./ml.) or di-isopropyl fluorophosphonate, DFP. (30 μg./ml.), were found to provoke considerable increases in the sensitivity of the isolated iris to acetylcholine.

There was some indication that eserine prolonged the second phase of the dual response of the isolated iris to 1000 μg./ml. or more of acetylcholine, in addition to its potentiating action upon both phases of this reaction. Larger doses of eserine than those referred to above caused a spontaneous constriction of the pupil, whereas the smaller ones were without any such direct action upon the iris.

The action of adrenaline

When adrenaline was applied to the isolated iris a brisk dilatation of the pupil was seen, the dilatatory phase being maximal within about 2 min. irrespective of the magnitude of the dose exhibited. The iris then gradually constricted to its original size, constriction taking longer when the dose was larger. The responses of the isolated iris to a series of doses of adrenaline are

illustrated in Fig. 6 which is a kymograph tracing made by the direct writing electromagnetic recorder. The isolated iris consistently gave small responses with as low a concentration of adrenaline as 0·01 μg./ml.

The galvanometer deflexions following each of a series of doses of adrenaline were plotted against time as with acetylcholine. The percentages of maximum action obtained with each of the doses applied to any one iris were then plotted against the logarithm of the concentration of the respective doses. Two examples of the graphs obtained are seen in Fig. 7 *a*, *b*.

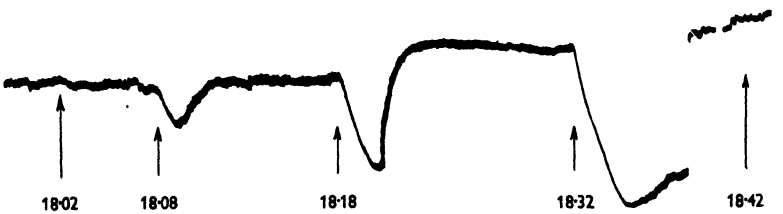

Fig. 6. A kymograph tracing made with the electromagnetic recorder illustrating the mydriatic effects of a series of doses of adrenaline containing 0·1, 1·0, 10 and 100 μg./ml. respectively. The total weights of adrenaline and the times of administration are indicated. The tracing is a continuous record except for a short break during the recovery phase with the largest dose of adrenaline. A downward deflexion indicates a dilatation of the pupil.

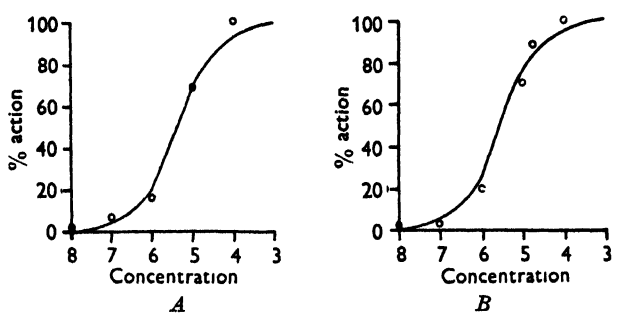

Fig. 7. Concentration-action curves when adrenaline is applied to the isolated iris. Presentation similar to that in Fig. 5. *A* and *B* are derived from two experiments not illustrated and demonstrate the concentration-action curves for adrenaline.

In a few rare instances moderate doses of adrenaline (1–10 μg./ml.) gave an unexpected constriction of the iris. These anomalous reactions could not be repeated and no procedure was discovered that would produce them at will.

DISCUSSION

It is of interest to compare the sensitivity of the isolated iris to drugs with that found by previous workers using isolated strips of the iris muscles. Velhagen (1934) obtained a contraction with a concentration of acetylcholine of $2{\cdot}5 \times 10^{-6}$ applied to an isolated strip of the rabbit's iris sphincter muscle. The isolated iris, however, responded to as low a concentration of acetylcholine as 0·001 μg./ml.

(1×10^{-9}), being about 2500 times more sensitive than Velhagen's preparation. Some isolated irides responded to even smaller concentrations of acetylcholine. It is possible that the trauma due to the dissection of an isolated strip of the iris sphincter muscle may account for its relative insensitivity to acetylcholine when compared with that of the isolated iris which suffers practically no trauma when studied by the method described above.

In favourable preparations, Poos (1926), using an isolated strip of the rabbit's iris dilator muscle, obtained a reaction with a concentration of 2×10^{-9} of adrenaline but other preparations appeared to require a concentration at least ten to one hundred times greater than this to produce recordable effects. With the isolated iris, many preparations readily gave a small dilatation to a concentration of 0·01 μg./ml. (1×10^{-8}) of adrenaline while almost every iris could be relied upon to dilate with a concentration of 0·1 μg./ml. (1×10^{-7}) of adrenaline (Fig. 6). Thus there appears to a close measure of agreement in the case of adrenaline sensitivity of the isolated iris of the cat and isolated strips of the rabbit's iris dilator muscle.

The dual nature of the reaction of the isolated iris to concentrations of 1000 μg./ml. or more of acetylcholine is of considerable interest for it bears a close qualitative similarity to the direct light reflex of the intact eye in which the pupil responds to a bright light impinging upon the retina by a rapid constriction that tends to overshoot the final pupil size for the new illumination. The overconstriction of the intact pupil under these circumstances may be a protective reflex designed to guard the eye against a sudden over-illumination. In view of the dual response of the isolated iris to a large dose of acetylcholine, it is possible that the overconstriction of pupil in the intact eye may be due to a peculiarity of the peripheral mechanism, the main feature of which is a similar dual response of the sphincter pupillae muscle as a result of a powerful stimulation via the third nerve resulting from urgent elicitation of the direct pupil reflex to light. However, before such a view could be accepted, it would be necessary to show that, in the isolated iris, the high concentration of acetylcholine required to elicit the dual response, the absence of the dual response with lower acetylcholine concentrations, and the relatively long time occupied by the response were all due to the reduced sensitivity of the iris, isolated in Ringer-Locke fluid, when compared with the iris in the intact eye.

A study of the concentration-action curves for acetylcholine (Fig. 5 *a*, *b*) and for adrenaline (Fig. 7 *a*, *b*) shows that the points fall, in general, very close to the hyperbola

$$Kx = \frac{y}{100 - y}.$$

These curves are similar to those described by Clark (1933) for the inhibition produced on frogs' isolated hearts and the contraction in frogs' isolated recti abdominis muscles brought about by acetylcholine. A useful measure for

comparison of the sensitivity of various preparations is the pI of the isolated iris which may be defined as the negative logarithm of that concentration of the drug tested that is required to elicit exactly 50% of the maximum reaction. This value can be determined with considerable accuracy from the concentration-action curves constructed for each iris. In the case illustrated, the pI for acetylcholine in Fig. 5 *a* is 5·2 and in Fig. 5 *b*, 6·1. In Fig. 7 *a*, the pI for adrenaline is 5·2 and in Fig. 7 *b*, 5·4.

Langworthy & Ortega (1943), in a histological study of the rat's iris, found prolongations of the ciliary body passing into the iris which consisted of arterioles coiled in the form of springs. They supported the view that the constrictive changes reducing the engorgement of these vessels played a part in the pupil dilatation mechanism. If such vascular changes played any large part in the cat's iris, they might be expected to modify the reactions of the isolated iris. However, with such preparations severed from the circulation, no evidence was obtained to support the work of Langworthy & Ortega.

This method permits quantitative studies of the drug reactions of the isolated iris, and also provides a means of rapidly testing the pharmacological activity of new compounds.

SUMMARY

1. A photoelectrical method of measuring changes in pupil area is described which permits quantitative studies of the action of a drug upon the cat's iris isolated in a perspex chamber through which oxygenated Ringer-Locke fluid flows. The method also provides a rapid and accurate means of determining the pharmacological activity of new compounds. Records were obtained using a galvanometer and/or a direct-writing electromagnetic device.

2. The reactions of the isolated iris have been studied in respect to acetylcholine, adrenaline, atropine, eserine and DFP.

3. In the case of acetylcholine and adrenaline, the concentration-action curves have been constructed and they can be fitted to the formula $Kx = \frac{y}{100-y}$. A useful measure for comparison of the sensitivity of the various preparations is the pI of the isolated iris which is defined as the negative logarithm of that concentration of the drug which is required to elicit exactly 50% of the maximum reaction. For acetylcholine the pI is about 6 and for adrenaline about 5·3.

4. It was common to obtain a constriction with as low a concentration as 0·001 μg./ml. of acetylcholine, and a dilatation with a concentration of 0·01 μg./ml. of adrenaline in the isolated cat's iris.

5. With concentrations of 1000 μg./ml. or more of acetylcholine, a dual constriction response was obtained with the isolated iris. The transient over-constriction of the pupil of the intact eye before it adopts the final size as a result of the direct pupil reflex to light is considered in relation to this dual response.

6. No evidence was obtained to support the view of Langworthy & Ortega (1943) that the constrictive changes in the engorgement of the spirally disposed arterioles of the iris played a part in the dilatation mechanism of the pupil.

I wish to thank Prof. R. J. S. MacDowall whose interest prompted a study of this problem and Dr D. B. Taylor for helpful discussion.

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THE RELATION BETWEEN FORCE AND VELOCITY IN HUMAN MUSCLE

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Light weights can be lifted more quickly than heavy ones. As the size of the weight is increased the speed of lifting becomes less and less, until eventually no movement takes place at all. As commonly observed, this phenomenon depends partly on the inertia of the object lifted, mainly on the inherent properties of muscle.

Isolated muscle exhibits the same behaviour when it is stimulated: the smaller the opposing force, the more quickly it shortens. Conversely, when allowed to shorten at a predetermined speed, for example in a Levin-Wyman ergometer, the force it exerts diminishes as the speed of shortening is increased. The maximal force is developed when the speed of shortening is zero, that is, when the contraction is isometric. Hill (1922) suggested that stimulation always brought about development of this maximal force, but that during shortening some of the force was used up in overcoming the viscous resistance of the muscle substance. Many experimental results were explained satisfactorily on this basis: for example, those obtained on the inertia flywheel (Hill, 1922; Lupton, 1922), and on the constant speed ergometer (Levin & Wyman, 1927).

In isotonic contractions the relationship between force applied and velocity of shortening was first investigated by Fenn & Marsh (1935). On the viscosity hypothesis the curve relating these two variables should be a straight line; yet they found in isolated muscles both of the frog and of the cat that it was clearly concave towards the origin (Fig. 2). They fitted an exponential equation to this curve (see p. 255).

A later investigation into the heat production during isotonic contraction (Hill, 1938) showed that the shape of the force-velocity curve was governed by the way in which energy was released during shortening: from thermal measurements Hill derived a simple equation relating the two variables

$$(P+a)(V+b)=\text{constant}=(P_0+a)b,$$

where P = force of contraction, V = velocity of shortening, a and b are constants,

and P_0 is the force exerted at zero speed, i.e. in an isometric contraction. He called this the characteristic equation, and it fitted the mechanical data of Fenn & Marsh, as well as the subsequent results of Katz (1939).

The importance of the force-velocity relationship is twofold. Its close connexion with the pattern of heat production is of theoretical significance; it is of practical interest also, since this relationship determines the mechanical behaviour of muscles loaded in different ways. Once the force-velocity characteristic is established, the variation of velocity in contractions against combinations of forces, inertias and elasticities can be predicted by ordinary mechanics.

For these reasons it seemed worthwhile to investigate the force-velocity relationship in the intact human subject, as suggested by Hill (1940) when he reviewed the inertia-wheel results of Lupton (1922) and found them compatible with a curved force-velocity relationship similar to that in frog's muscle. The first direct investigation of the problem was made by Dern, Levene & Blair (1947), whose results are discussed below (p. 276).

The choice of a movement. Very few movements lend themselves to quantitative study. To be suitable a movement should satisfy the following criteria:

(1) The joint should be geometrically simple.

(2) The movement should involve few muscles, which should have small origins and insertions.

(3) The movement should not disturb rigid fixation of the rest of the body, and should lend itself to graphic registration.

(4) The movement should be accurately reproducible. This is easiest to achieve if only slight skill is involved.

All these criteria are substantially satisfied by the movement of flexion of the elbow, made with the upper arm abducted at right angles to the body, and in a line with the shoulders; the forearm supinated and moving in a vertical plane. In every instance the movement was made with as much speed and force as the subject could muster. In the inertia wheel experiments mentioned above the same movement was used.

Maximal movements can be repeated with surprising consistency; this is dealt with in detail later. Consistency is maintained, not only throughout a single experiment, but over a period of many months.

Interpretation of results obtained on movements of the whole forearm in terms of the properties of the five flexor muscles is attended by two major difficulties, which are (*a*) geometrical and (*b*) nervous.

(*a*) The origins and insertions of the five muscles are not arranged in any simple way relative to the long axes of the fore- and upper-arms. Consequently, the relationship between the tension in a given muscle, and the torque which that muscle produces on the forearm as a whole, is a complicated function of the angle of flexion, and this function is different for each of the five muscles.

However, as is shown later, only a small error is introduced by assuming that the five muscles are all horizontal when the arm is held in the experimental position. This being so, the horizontal components of the forces and velocities measured at the hand will be proportional to the actual forces and velocities of contraction of the muscles themselves. The experiments relating force to velocity have been made with this picture in mind. The forces and velocities which have been measured are all horizontal components measured at the hand.

(*b*) It seems to be agreed that even in the most violent effort not all the muscle fibres are fully active at once; but it is not known whether the amount of excitation is constant in such a maximal movement, or whether it depends, on the position of the limb, for example, or the force against which the movement is made. ('Excitation' is meant to express both the number of muscle fibres involved and the frequency of discharge in them.) There is at present no method for determining the degree of excitation in an intact human subject: no satisfactory basis has been demonstrated for the exact quantitative interpretation of electromyograms. All the experiments described in this paper support the hypothesis that the muscles in the body follow a fixed force-velocity relationship in contractions against different types of load, a result which can be accounted for in either of two ways:

(1) That the degree of excitation is constant, and that the muscles themselves follow a fixed force-velocity relationship.

(2) That the degree of excitation varies with the force, but in such a way that for each value of the force, no matter how other conditions alter, only one value of the velocity is possible. This would make the force-velocity characteristic as much a property of the central nervous system as of the muscles.

There is no objective way of distinguishing between these alternatives, but the first seems more probable, for all the studies on isolated muscles indicate that they themselves follow fixed force-velocity relationships.

SECTION I. EXPERIMENTAL DETERMINATION OF THE RELATION BETWEEN FORCE AND VELOCITY IN HUMAN MOVEMENT

In this group of experiments the subject pulled against different weights and the velocity of his hand was measured at the end of each movement.

METHODS

The apparatus (Fig. 1) consisted of a triangular oak lever whose axle ran freely in self-centring ball bearings, which were mounted on blocks at the end of a table. The subject pulled on this lever through a Bowden wire cable in which the tension was varied by varying the suspended weight. The handle attached to the cable was free to rotate about a horizontal axis. The dimensions of the lever were: total length, 39·5 cm.; length of short arm, 7·4 cm.; moment of inertia, 95800 g.cm.^{2}.

The subject kept his upper arm fixed during each movement by pressing it up against a padded block of wood screwed to the table. Movement of his body was prevented by a vertical board at the end of the table. In order that the force applied to the arm shall be constant throughout each

movement the cable must remain horizontal. From Fig. 1 it is clear that this will happen automatically provided that the length of lever used is the same as the radius of rotation of the forearm and that its axle is the same height above the table as the centre of rotation of the elbow.

The radius of rotation of the forearm is easily measured by having the subject grasp a pencil while his forearm is swept passively over a sheet of paper. Some care is needed to prevent movement of his upper arm during the process. The circle drawn is remarkably perfect, the variation in radius being less than 1 % for a movement of more than 90°. The height of the centre of rotation of the elbow is then the difference between the radius of rotation and the height of the handgrip above the table when the forearm is vertical.

The velocity of each movement was estimated from the charge accumulated on a condenser. As the lever rotated it swept a light spring over the surface of an ebonite sheet, making contact for a brief interval with a recessed brass strip. Contact was made and broken sharply, so the velocity

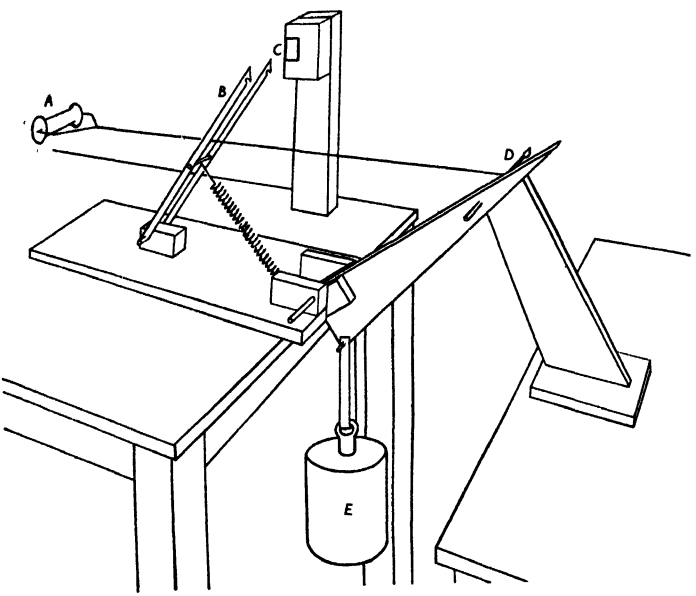


Fig. 1. Isotonic lever for human subjects. *A*, handgrip attached to cable; *B*, catch to hold lever up at the end of movement; *C*, fixed contact; *D*, lever with moving contact; *E*, weight.

of movement could be calculated from the duration of the contact. As soon as contact was closed a condenser began to charge through a resistance. The total charge, Q, which it accumulates, is related to the duration of contact, t, by the expression

$$t = RC \log_e \frac{Q_0}{Q_0 - Q},$$

where R = resistance in ohms, C = capacity in farads and Q_0 = charge when t is very long. Q and Q_0 were measured by discharging the condenser through a ballistic galvanometer. In practice it was found convenient to include a second key in the circuit, in order to discharge the condenser without passing current through the galvanometer; for even with well-insulated circuits (leakage resistance 1000 MΩ.) enough charge leaked across the open contacts to require removal before each determination.

Range of movement. (All angles are measured on the proximal side of the forearm, i.e. full extension is 180°.) The load was supported by a stop so that the lever was at 140° with the horizontal before each movement. At the end of the movement (75°) the load was held up by a spring catch.

The fixed contact was arranged to measure the velocity when the arm was at an angle of 80° with the horizontal. Subsequent experiments have shown that maximal effort does not cease until the arm reaches 70°; nor is there appreciable evidence of antagonist activity before this point.

Measurement of isometric tension. P_0, the isometric tension, appears in the characteristic equation. It was measured by a simple spring balance, with the forearm at an angle of 80° with the horizontal, i.e. in the position at which the velocity was measured.

Procedure. Preliminary experiments on two subjects showed that satisfactorily consistent results were obtained and these yielded a curve of force against velocity similar in shape to those found by Fenn & Marsh (1935), Hill (1938) and Katz (1939) on isolated muscles. A large-scale experiment was therefore carried out to determine more precisely the relationship between force and velocity in one subject.

Units. All forces are given in megadynes, where 1 megadyne $= 10^6$ dynes or approximately 1 kg.wt.

Arrangement of experiment. The tension at the hand was varied in eleven steps from 0 to 15·23 megadynes and at each step thirty measurements of the velocity were made, 330 determinations in all. Only five velocity measurements at the same tension were made at any one time and each one followed a rest period of at least a minute; fatigue was thus avoided. The order in which the sixty-six groups of five determinations were made was decided from random number tables and the whole experiment was spread over a period of 2 weeks. This arrangement was adopted in order that each group of measurements should represent a true random sample from the population of such measurements, to which sampling theory might be legitimately applied.

RESULTS

The thirty velocity measurements made at each tension were in every case distributed normally about their mean value. Even in the worst distribution the shape constants $\sqrt{b_1}$ and a, which measure skewness and flatness respectively (Geary & Pearson, 1938), were within normal limits:

$$\sqrt{b_1} = 0{\cdot}08 \qquad P \gg 0{\cdot}05,$$
$$a = 0{\cdot}862 \qquad 0{\cdot}10 > P > 0{\cdot}05.$$

The experimental curve of mean velocity against tension is plotted in Fig. 2. As the experimental relationship between force and velocity was now established with known precision, it remained to be seen whether this could be described by the characteristic equation. Since that can be written

$$\left(\frac{P}{a}+1\right)\left(\frac{V}{b}+1\right) = \frac{P_0}{a}+1,$$

the shape of the characteristic curve is fully defined by the ratio a/P_0.

After a few trials with different values of a/P_0, it became clear that the experimental results were *not* fitted by the characteristic equation, except at tensions greater than about 0·3 P_0. However, the experimental results as they stand are not engendered by the muscles alone; they result partly from the properties of the apparatus. It seemed likely that the inertia of the apparatus and forearm might so diminish acceleration that the full velocity could not be reached before the movement was completed. This effect would naturally be greatest when the final velocity was greatest, and might account for the observed departure from the characteristic equation. The forearm provides most of the inertia when only small weights are lifted, so there is little point in improving

the apparatus. Instead, allowance is made mathematically for the effect of inertia.

Correction for inertia of apparatus and arm. Imagine that the contractile part of the muscle is pulling against the inertia of a mass M as well as a constant force F. The force which the muscle can exert at any point is some function, as yet unknown, of its velocity of shortening at that point; let us say $P=f(V)$. But, in the case considered,

$$P=F+M\frac{dV}{dt}, \quad \text{so} \quad f(V)=F+M\frac{dV}{dt}.$$

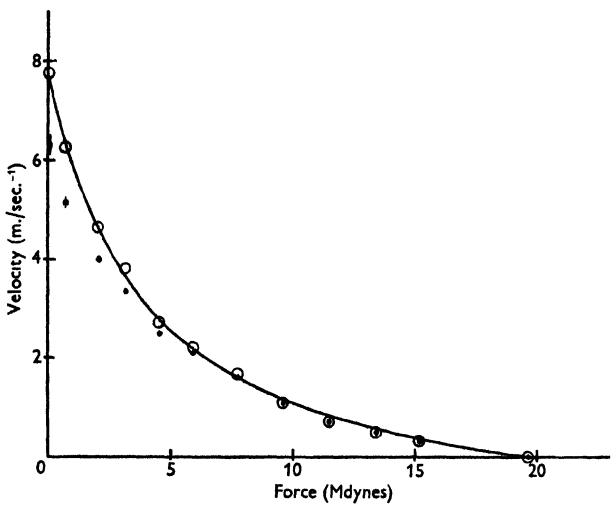


Fig. 2. Experimental relation between force and velocity (subject D.W.). Dots: means of thirty determinations of velocity. Six times the standard error is plotted as a vertical bar through the mean. At many points it is too small to be visible on reproduction. Circles: experimental points after correction for inertia. Curve drawn from $(P\times a)\,(V\times b)=(P_0\times a)b$ with $a/P_0=0{\cdot}20$.

The solution of this equation will describe how the velocity of the system rises during the movement, thus making it possible to decide whether inertia does limit acceleration in the way suggested. The immediate problem was to discover whether the characteristic equation represents the true force-velocity relationship in the muscle and this was done by substituting

$$P=\frac{(P_0+a)\,b}{V+b}-a \quad \text{for} \quad P=f(V)$$

in the above equation. Then

$$\frac{(P_0+a)\,b}{V+b}-a-F=M\frac{dV}{dt}. \tag{1}$$

The theoretical effect on acceleration was then compared with the effect observed experimentally.

The method for finding the correct values of a and b is somewhat involved, as they should be based on the $P:V$ curve *after* correction for inertia. In Appendix A an expression is derived for the amount by which the actual velocity at the end of the movement falls short, owing to inertia, of the theoretical final velocity. It involves F and P_0, which are already known; M, which, as shown below, can be measured; x, the measured length of pull and the constants a and b, which it is required to find. The true values of a and b can then be calculated from the experimental $P:V$ curve in the following way:

(1) Provisional values of a and b are estimated by fitting a curve directly to the experimental data.

(2) With these and the known values of F, P_0 and M the velocity deficit is calculated for each experimental point. When each deficit is added to its experimental point a new set of points above the first is obtained.

(3) Fitting a new characteristic equation to this set of points yields new values of a and b, with which the whole cycle is repeated.

(4) After three or four repetitions there remains no discrepancy between the corrected points and the characteristic equation from which they are derived. These final values of a and b are therefore the correct ones.

The magnitude of the correction involved can be seen from Figs. 2 and 3, where corrected experimental points are plotted with their appropriate theoretical curve. It is clear that the characteristic equation gives a good description of the corrected experimental results. Of course, other equations may do just as well; for example, that derived by Fenn (1938)

$$P = P_0 e^{-aV} - kV,$$

in which a and k are constants. It is the shape of the curve which is important, while the equation used to describe that shape is largely a matter of convenience.

The experiment was not done in such detail on the other four subjects, and each point is based on five instead of thirty determinations of velocity. Each was corrected for inertia in the way described above. Their fit with the characteristic equation is in every case quite good, and the range of values of a/P_0 is approximately the same as that reported by Katz (1939) in the frog. It is also interesting to note that, although the maximal force exerted varies from 12 to 20·5 megadynes, the maximal velocity attained is relatively constant, a finding reported previously by Dern *et al.* (1947). This result might have been anticipated on dimensional grounds. Animals of different size, but similar shape, greyhounds and race-horses for example, have the same maximal running speed.

The results on the five subjects are collected in Table 1.

Determination of inertia. The inertia M whose value was used in the previous calculation consists of three parts, all of which should be expressed in terms of their equivalent mass at the hand.

(1) The equivalent mass of the weights is found by multiplying their mass by $(S/L)^2$, where S and L are the short and long arms of the lever.

(2) The moment of inertia of the lever itself is most easily found by measuring the moment m which it exerts when horizontal, and also its natural period of oscillation T. Then, moment of inertia $I = 4\pi^2 T^2 m$, and equivalent mass $= I/L^2$.

(3) The moment of inertia of the forearm can be determined in several ways. Braune & Fischer (1894) suspended the limbs of cadavers on steel pins and measured their moment and period. The same principle was used by Hill (1940), with his own completely relaxed forearm. Fenn (1938) measured the initial

TABLE 1. Force-velocity characteristics of five subjects

	Subject	Age	Sex	P_0	V_0	$a/P_0 = b/V_0$
1	J.H.	22	F.	12·0	670	0·33
2	L.M.	24	M.	20·0	700	0·48
3	R.S.	20	F.	12·0	650	0·42
4	M.R.	22	M.	20·5	600	0·37
5	D.W.	23	M.	19·6	775	0·20

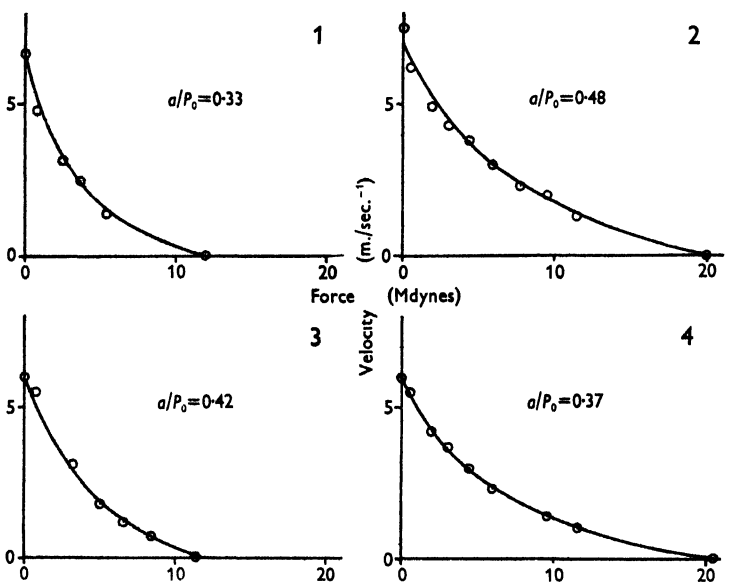


Fig. 3. Force-velocity characteristics of four subjects. Circles: experimental points corrected for inertia. Curve drawn from the characteristic equation.

acceleration following sudden application of a known force, and his results were of the same order of size as those of Braune & Fischer, that is, about 0·5 kg.cm.^{2}.

In the present experiments a direct method was used. The forearm, with the fist held in the experimental position, was lowered by 2 cm. steps into a tall jar of tepid water. Each segment of the arm displaced its own volume of water over into a beaker, where it was weighed. The best device for collecting all the displaced water was found to be an open-ended siphon with its outlet a few inches below the rim of the jar; care was required to avoid breaking the siphon by even a slight withdrawal of the arm. This arrangement was very sensitive

and reliable: when 10 c.c. of water was added with pipette to the jar, this volume was always recovered to within 0·2 c.c. from the beaker.

The total volume of each 2 cm. segment of forearm being known, the proportion of bone was found by repeating the procedure with a skeleton chosen to match exactly the radiograph of the subject's arm. The mass of each segment was then calculated on the assumption that the density of soft tissues was 1 g./c.c.; while that of the bones was found to be 1·4 g./c.c. after repeated heating and cooling under water had removed air bubbles. Then the moment of inertia $=\Sigma M_n r_n^2$, where M_n is the mass of the nth segment, distant r_n from the joint axis.

Since all forearms are of similar shape the inertia was not determined directly on the other four subjects, but was calculated from that of subject D.W., multiplying by $\left(\frac{\text{length of forearm of subject}}{\text{length of forearm of subject D.W.}}\right)^5$.

For subject D.W., moment of inertia $=0{\cdot}53$ kg.cm.^{2}, equivalent mass at 32 cm. $=0{\cdot}52$ kg., maximum moment $=21{\cdot}9$ megadyne-cm.

The variation of equivalent mass with position. From the point of view of the flexor muscles the equivalent mass of the forearm is not the same in all positions of the limb. The equivalent mass of the forearm is only equal to (moment of inertia)/r^2 when the angle of flexion is 90°. At any other angle θ the equivalent mass is greater by the factor $1/\sin^2\theta$. The variation is small (13%) between 70 and 110°, but increases steeply outside these limits, so that at 50 and 130° the discrepancy has reached 69%. Fortunately, in the slower movements where the early part of contraction is of particular interest, only a small part of the total inertia is provided by the arm; the equivalent mass of the weights does not vary with the position of the limb.

SECTION II. THE RISE OF VELOCITY IN ISOTONIC CONTRACTIONS

The method of correcting for inertia used in the previous section is based on the assumption that one can predict mathematically how the velocity will rise in a single contraction. The only property attributed to the muscle and its central nervous connexions was that at any instant its velocity of shortening should be some definite function of the tension which it exerted. It was shown that the characteristic equation gave a good approximation to this function.

However, it remained to be seen whether the velocity did rise in the way predicted; which involved measurement of the velocity throughout the movement and not just at one point as in the previous experiments.

METHODS

Three different methods were used to register the variation is velocity during single contractions.

The first method used was very simple and quickly set up. A small electromagnet attached to the lever was energized by current from the mains. In this way a small pointer was caused to vibrate at 100 c.p.s. in a direction parallel with the long axis of the lever. As the subject pulled,

the pointer drew out the arc of a circle on a piece of smoked glass held vertically in a Palmer stand. On this arc were superimposed undulations 10 msec. apart. After varnishing the record the distance was measured between each undulation and the starting position. The increment in displacement is clearly proportional to the average velocity during that period.

In the second method used, high-speed cine-films of the movement were taken, and the horizontal movement of the hand between frames measured. A clock and a scale of decimetres were included in the picture to provide an absolute calibration in terms of velocity.

Both methods suffer from two defects. The distances to be measured are small, so slight inaccuracy causes marked scatter of the points representing velocity: also both are time-consuming, reducing the number of experiments performed. A more elaborate and accurate apparatus was therefore devised.

A small coil of 100 turns was mounted on the axle of the lever as shown in Fig. 4 so that it rotated in the gap between the poles of a powerful magnet. The voltage induced depended on the speed of rotation of the coil.

The magnetic flux linked with coil is

$$N = HAn \sin\theta,$$

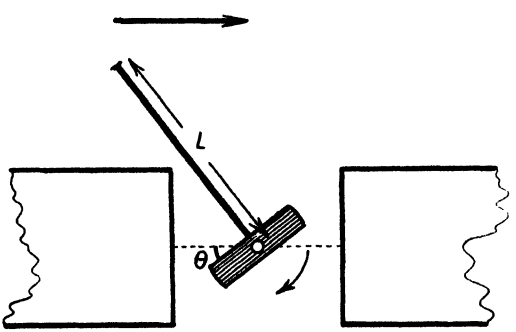


Fig. 4. For explanation see text.

where H = magnetic field, A = area of coil, n = number of turns and θ = angle between axis of coil and the magnetic axis; for the voltage developed is

$$E = -\frac{dN}{dt} = -HAn\cos\theta\frac{d\theta}{dt},$$

and the horizontal component of velocity of hand is $V = L\cos\theta\frac{d\theta}{dt}$, where L = length of lever. Therefore

$$E = -\frac{HAn}{L}\times V.$$

That is, a voltage is developed which is proportional to the horizontal component of the velocity of the hand.

In order to test for non-uniformity in the magnetic field, the coil was detached from the lever and rotated at constant speed by a small electric motor. The alternating voltage developed was amplified and applied to a Wien bridge adjusted to remove the fundamental frequency. The residue of harmonic had 1% of the original amplitude.

To register the velocity of the hand, current from the coil was passed to a Downing galvanometer (period 12 msec., sensitivity $1{\cdot}66\times10^{-7}$ amp./mm. at 1 m., resistance 50 Ω.), whose deflexion was recorded on a falling-plate camera. It was arranged that the galvanometer should be always critically damped, and a series resistance of 100–1000 Ω. was included to limit the current drawn from the coil. Time marks were provided every 20 msec. by a stroboscopic lamp.

At one point the absolute velocity was measured by the condenser method already described, to provide a calibration on each record. This point was identified on the trace by a bright flash from

the stroboscopic lamp, which was triggered from the condenser circuit, but did not withdraw any current from it.

In later experiments the velocity-time curve was displayed on a long after-glow cathode-ray tube, using a single-stroke time-base and a d.c.-connected Y amplifier with a gain of about 3000. This is by far the best experimental arrangement.

RESULTS

The subjects pulled with maximal effort against a succession of weights in exactly the same way as before. A typical set of velocity-time curves is shown in Fig. 5A.

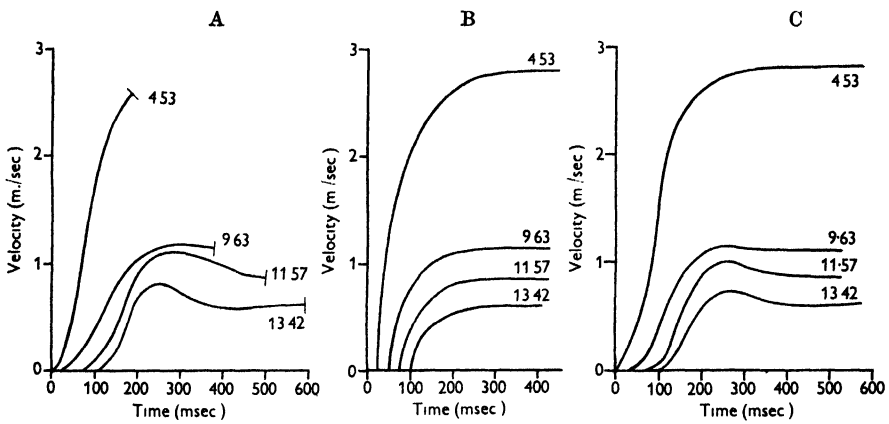


Fig. 5. Velocity-time curves. Subject D.W. A, Experimental curves. The bar at end of each curve marks the point at which lever hits catch, just after its velocity has been measured for calibration. Subsequent rapid and irregular fall in velocity not shown. B, Theoretical curves. Calculated from $(P_0+a)b/(V+b)-a=F+MdV/dt$ (see Appendix A). C, Theoretical curves. Calculated electrically from equation 2, p. 261. Figures on graphs indicate tension (megadynes) against which pull was made.

The theoretical curves, derived from

$$\frac{(P_0+a)\,b}{V+b}-a=F+M\,\frac{dV}{dt},$$

are shown in Fig. 5B. The integrated form of the equation, from which the points are actually calculated, is given in Appendix A.

As expected, when the tension is small, e.g. $F=4{\cdot}53$ megadynes, there is not time enough to reach a steady velocity before the movement is brought to a stop. This illustrates the importance of the inertia correction used in section I. However, it is clear that although each theoretical curve does rise to the right final velocity, the *mode of rise* is rather different from that found experimentally:

(1) The calculated curves rise sharply from the base-line. Experimentally each begins more gently, with a distinct concavity upwards. The reason for this concavity was thought to be that all the motor units did not become active at once. Fenn (1938) suggests that excitation takes about 40 msec. to reach its

maximum, and this agrees quite well with the duration of the concavity. An alternative explanation is given below.

(2) Experimentally, when the force is large, e.g. $F = 11{\cdot}57$ megadynes, the velocity is seen to rise to a peak before settling towards its steady level. When the force is larger still, e.g. 13·42 megadynes, the velocity executes a damped oscillation about its final value. These observations have been repeated consistently in ten subjects. To explain them, three possibilities were considered.

(1) *Flexion of the wrist might occur suddenly, early in the movement.* The extra shortening represented by the peak of the velocity-time curve is equal to $\int V\,dt$, that is, the area of the peak. This is only about 4 cm. with even the greatest peaks, so, if it occurred quickly enough, flexion of the wrist could account for the observed result.

The question was investigated by photographing the movements with a high-speed camera, kindly made available by Messrs Kodak. The films showed that there was some flexion of the wrist early in the movement, but that most of it took place after the velocity peak was over. Moreover, the shape of the velocity-time curve was not altered when the wrist was held in full flexion throughout the movement by a light plaster-and-metal splint.

(2) *The degree of excitation might vary during the movement.* If the degree of excitation to a muscle alters, its isometric tension, P_0, will be changed. Now in the characteristic equation P_0 appears as a constant. If, therefore, it is shown that the same equation applies at every instant during a contraction, P_0 must remain constant and there can be no variation in the degree of excitation.

Direct investigation of nervous activity was therefore postponed until it had been decided whether or not the characteristic equation did apply throughout the movement. That it did became clear on considering the third possibility.

(3) *Oscillation might result from the presence of an inert elastic element between the contractile part of the muscle and its load.* The presence of such an element in isolated muscle was first demonstrated by Levin & Wyman (1927). Hill (1938) showed that a dual structure consisting of 'characteristic' elements in series with inert elastic ones could also explain the effects which they attributed to visco-elasticity.

Measurements of heat production also supported the hypothesis. The amount of mechanical energy supposedly stored in the elastic element at the peak of contraction could be calculated: it was found to be of the same order of size as the measured heat of relaxation. The heat was therefore considered to be merely degraded mechanical energy. More recently it has been shown (Hill, 1949) that when the conditions of shortening are arranged so that no mechanical energy is stored, no relaxation heat is found.

In human experiments the presence of such an element was suggested by the observation that peaking and oscillation in the velocity-time curve was

increased when a spring was incorporated in the cable. The mechanical system visualized was that shown in Fig. 6. During the initial acceleration energy is stored in the elastic element, to be released again as the velocity approaches its steady value. The way in which the velocity of such a system should rise can be predicted by simple dynamics. The point to be tested is whether prediction is similar to experimental fact. In Fig. 6: F = isotonic force, P = tension in muscle, v = velocity actually measured, V = velocity of shortening of muscle, G = compliance (strain/stress) of elastic element:

Equating forces, $P = F + M\frac{dv}{dt}$. Equating velocities, $V = v + G\frac{dP}{dt}$. But $V = \frac{(P_0+a)\,b}{P+a} - b$, from the characteristic equation. Combining these equations to eliminate P and V

$$v + GM\frac{d^2v}{dt^2} = \frac{(P_0+a)\,b}{F + M\,(dv/dt) + a} - b. \tag{2}$$

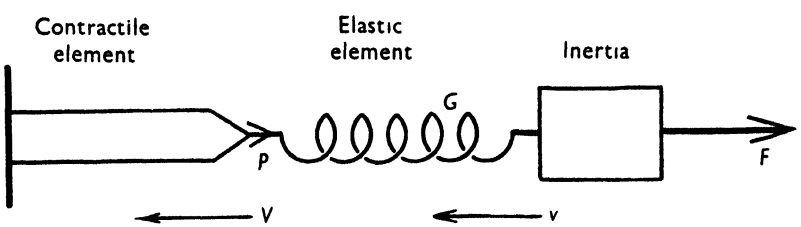


Fig. 6. Proposed structure of muscle pulling against a load.

This non-linear differential equation cannot be integrated; that is, it is not possible to obtain from it an expression without differentials giving v in terms of T. A numerical solution must be obtained in each individual case by arithmetical integration.

Two examples worked out in this way looked similar to experimental curves, but in order to test the theory fully it was obviously necessary to compare calculated and experimental results under a variety of conditions.

In practice there was an obstacle to carrying out this plan: arithmetical integration is an excessively laborious and time-consuming process. An electrical method for performing the calculations, described elsewhere (Wilkie, 1950) was therefore developed.

Of the parameters of the equation; F, P_0, M, a and b are all known from section I, while an independent method of calculating G from measurements of isometric tension will be described below. (Section III.)

The variation of compliance with tension has not been taken into account, the central value at 0·67 P_0 being used (Table 2). That this makes little difference to the shape of the velocity-time curve was shown by arithmetical

solutions using linear and non-linear compliances. The result would be expected, since the tension varies only slightly in each isotonic contraction.

The experimental (*A*), and calculated (*C*), curves are shown in Fig. 5. The similarity between the shapes of the two sets of curves supports the hypothesis that there is an inert series elastic element between the contractile part of the muscle and its load, and that the characteristic equation is obeyed throughout the movement.

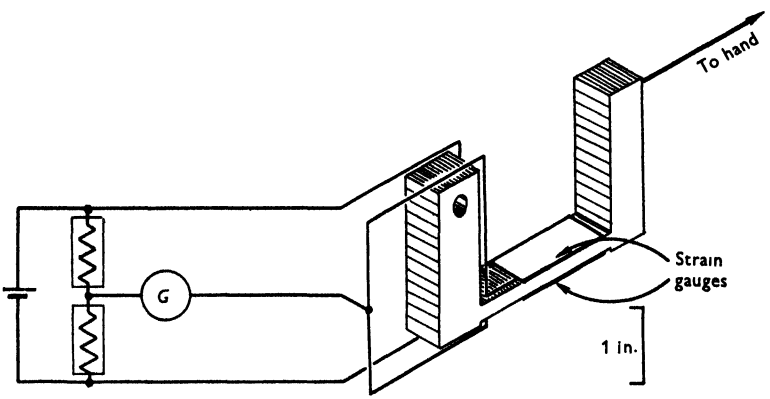


Fig. 7. Arrangement of resistance strain gauges and associated circuit for measurement of isometric tension.

The initial concavity is accounted for as well as the peaking, so that it is unnecessary to suppose that the maximal excitation develops only slowly. Indeed, the characteristic equation is obeyed so well throughout the movement that there cannot be any large variation in the degree of excitation.

The inertia correction used in section I is barely affected by these new findings, for it is only applied when the tension is small and 'peaking' is not evident. With large tensions the peak has disappeared long before the velocity is measured.

SECTION III. THE RISE OF TENSION IN ISOMETRIC CONTRACTIONS

The force developed in an isometric contraction requires an appreciable time to reach its final value.

In order to explain the slow rise in tension, Hill (1938) made the assumption that muscle is a two-component system consisting of actively contractile and passively elastic structures in series. During isometric contraction the active elements were imagined to shorten at the expense of the passive ones, the total length of muscle remaining constant. His conclusions were confirmed experimentally by Katz (1939).

Apart from their intrinsic interest, isometric experiments are important in

providing an independent means for estimating the series compliance, G, of the elastic element.

If one assumes that the relationship between force and velocity in the contractile elements is the same under isometric as it was under isotonic conditions, the compliance of the series element can be calculated from the rate of rise of isometric tension.

Let tension exerted by muscle $= P$, series compliance $= G$. Then, rate of shortening of active element is

$$V = \frac{(P_0 + a)\,b}{P_0 + a} - b.$$

Rate of lengthening of passive element $= G\,dP/dt$. Equating these

$$G = \frac{(P_0 + a)\,b}{P + a} - b \Big/ \frac{dP}{dt}.$$

The constants in this equation are all known from section I: values of P and dP/dt were obtained from experimental tension-time curves.

METHOD

In preliminary experiments the isotonic lever was replaced by a stout bracket of spring steel, bolted down to the table. The cable and handle were attached to its free end and the deflexion of the bar magnified by an arrangement of mirrors giving a light path of 3 m. Records of the displacement were made by a falling-plate camera.

Such apparatus is easily set up, and has a suitably short period of vibration (31 msec. in this case), but it is difficult to mount the spring so rigidly as to prevent relative movement between it, the optical system and the camera.

A far more convenient and adaptable tension-measuring device was built with resistance strain-gauges, each of which contains a grid of fine wire whose electrical resistance is increased on stretching. A pair of gauges (British Thermostat Company, SE/A/11, 2500 Ω.) were mounted on a piece of mild steel of the shape shown in Fig. 7. They formed two arms of a Wheatstone's bridge, which was completed by a second pair of gauges. The degree of unbalance of the bridge was indicated directly on a Downing galvanometer (period 50 msec., sensitivity 8×10^{-9} amp./mm. at 1 m., resistance 100 Ω.).

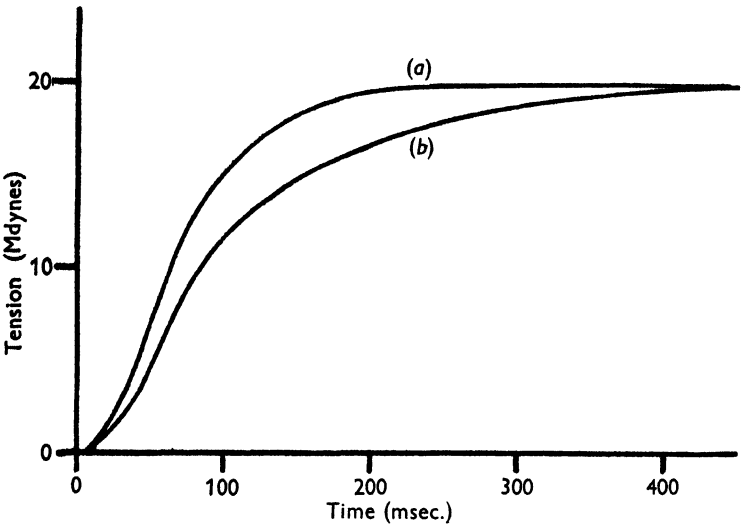
The advantage of this paired arrangement of gauges is that temperature effects are neutralized; also the sensitivity to tension is doubled while the response to twisting is reduced.

The calibration curve, which was a straight line, was based on six measurements at each of twenty-two different tensions. The average standard deviation within the groups of six was only 0·58% of the half-scale deflexion.

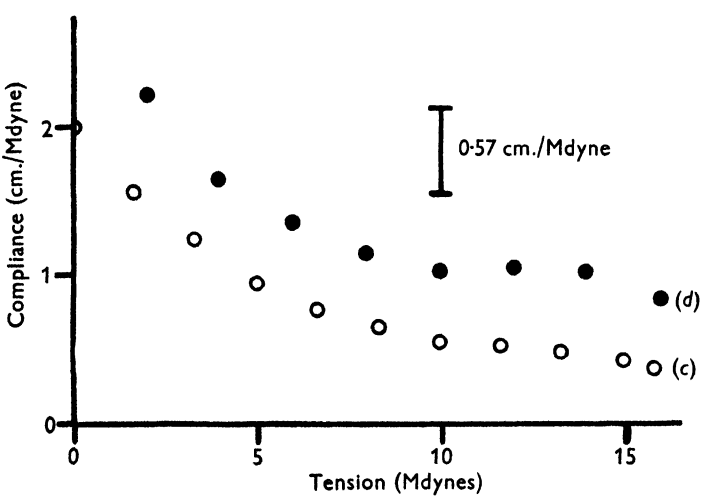
The tension gauge was mounted on the isotonic lever, which was held vertical by a rigid strut. The subject pulled from the far end of the table, exactly as in the previous experiments.

RESULTS

The relationship between isometric tension and time in subject D.W. is shown in Fig. 8A, curve a, which is the mean of five independent observations. From this figure the value of dP/dt was found at chosen intervals of P, by drawing the geometrical tangent to the curve. Knowing dP/dt and P at a number of different points on the curve, the compliance at these points was calculated from the equation above.



A



B

Fig. 8. A, The rise of isometric tension with time. Subject D.W. (*a*) arm alone; (*b*) with compliance of 0·57 cm./megadyne added to cable. B, The variation of compliance with tension; calculated from curves of A. (*c*) arm alone; (*d*) compliance of 0·57 cm./megadyne added to cable. Note the approximately constant vertical displacement between the curves.

The result of this calculation is shown in Fig. 8B, curve *c*, where it is seen that the compliance diminishes as the tension increases. This behaviour is similar to that of resting muscle; or of a fibrous structure, a knitted stocking for example, whose fibres do not all run in the same direction or do not all start tight.

However, if the maximal excitation takes an appreciable time to become established, the resulting slow rise in tension will also appear as an increased compliance when the tension is low, indistinguishable from a true increase in compliance. In order to provide independent evidence on this point the experiment was repeated with a spring (compliance 0·57 cm./megadyne) in series with the cable.

The effect of added compliance. The new tension-time curve, *b* in Fig. 8A, rises more slowly than before. From it is calculated the compliance-tension curve *d* in Fig. 8B. This curve is found to be displaced vertically by an amount corresponding approximately to the known added compliance. Now if the curvature of Fig. 8C were due to the slow increase of excitation, this curvature should be diminished by increasing the series elasticity; for a given tension would then be reached later in the contraction, after the

TABLE 2. The series compliance measured at the hand. Values correspond to a tension of 0·67 P_0

Subject	J.H.	L.M.	R.S.	M.R.	D.W.
Compliance (g.cm./megadyne)	1·1	1·1	0·6	3·6	0·5

excitation had become steady. As in Fig. 8C and D are the same shape, it is likely that this is determined by the muscle compliance. The series compliance was determined in the same way on the other four subjects, and the results are shown in Table 2. The compliance varies with the tension, but only one value is given in the table, corresponding to $P = 0{\cdot}67 \times P_0$. The effect of adding extra compliance (0·57 cm./megadyne) to the cable as described above, was to produce a displacement of 0·4–0·7 cm./megadyne in the compliance-tension curve.

The values given in Table 2 seem surprisingly large, but it should be remembered that they were calculated as though the compliance were localized at the hand. Variation in length in the muscles is magnified approximately seven times by the lever of the forearm and their force is diminished by the same ratio; so the compliance measured on isolated muscle would be only $(1/7)^2$, or about 2%, of the value shown in the table.

The anatomical site of the elastic element is a matter of speculation. It seems unlikely that all the compliance resides in the tendons, which are relatively rigid structures. Possibly the muscle fibres themselves contain series elastic elements, which one is tempted to identify with the isotropic disks of the myofibrils, for Buchthal & Lindhard (1939) have shown that these become stretched during isometric contraction by active shortening of the anisotropic

disks. However, there is at present no other evidence for this view than superficial resemblance.

By integrating under the compliance-tension curve it is possible to calculate the total lengthening of the elastic element under full isometric tension. In the case of subject D.W., Fig. 8B, allowing for the lever ratio of the forearm, this corresponds to about 10% of the resting length of the muscle. In isolated muscles the ratio is regularly in the same region, from 10 to 15%.

SECTION IV. THE INTERPRETATION OF MOVEMENTS IN TERMS OF MUSCLES

The experiments of the preceding sections have shown that in maximal voluntary movements there is a definite relationship between velocity of movement and force exerted, both being measured horizontally at the hand.

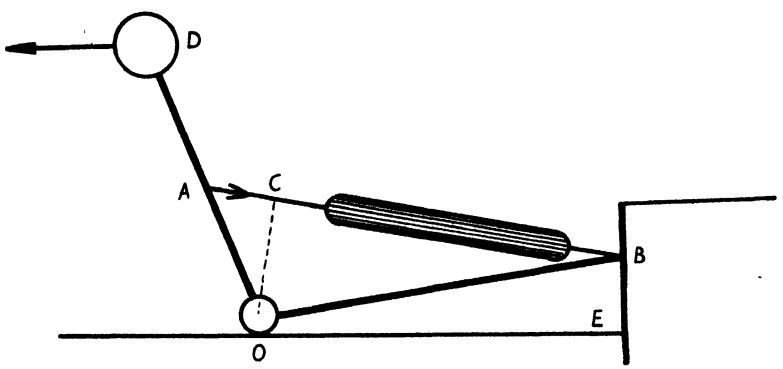


Fig. 9. Diagram of fore- and upper-arms showing the action of a single flexor. *B* and *A*, origin and insertion; *O*, elbow; *D*, hand-grip. Note that the humerus is not horizontal.

This relationship applies at every instant during the shortening, and it can be represented by the characteristic equation. Whether the properties found experimentally on whole movements are also the properties of individual human muscles has already been discussed, and throughout this paper the two have been tacitly identified in order to simplify explanation.

As indicated earlier, it will be necessary to deal with the mechanical and nervous aspects of the problem separately.

The mechanics of the elbow joint

The action of the five flexors of the elbow joint was analysed in considerable detail by Braune & Fischer (1890), who set out to discover what contribution each muscle made to the total torque at the elbow. At 5° intervals of flexion they measured the equivalent lever arm of each muscle, that is, the perpendicular distance from muscle to joint axis. Subsequently, they calculated the equivalent lever arm trigonometrically, using the measured distances of origin and

insertion from the joint axis; this calculation agreed well with their direct measurements.

The agreement is of importance, since a similar calculation is used in this paper to determine the ratio between the tension in each muscle and the horizontal force which that muscle produces at the hand. It has been assumed up to this point that the lever ratio was constant throughout flexion. That this is actually the case is shown below.

Consider one muscle. It is required to calculate OC, the equivalent lever arm (see Fig. 9).

$$\text{Equivalent lever arm} = OC = \frac{OBOA \sin AOB}{\sqrt{(OA^2 + OB^2 - 2OAOB \cos AOB)}},$$

and

$$\text{lever ratio} = \text{horizontal force/tension in muscle} = \frac{OC}{OD \sin AOE}.$$

The lever ratio has to be calculated for each muscle over the whole range of shortening used experimentally. This involves measuring AO and OB for each muscle.

TABLE 3. Distances from joint axis in centimetres

	M.R.		D.W.		Braune & Fischer Means of four subjects	
Subject ...	OA	OB	OA	OB	OA	OB
Pronator teres	13·50	1·70	11·35	1·38	11·35	1·37
Extensor carpi radialis longus	24·00	3·00	25·11	2·90	22·05	3·50
Brachialis	2·70	9·00	3·14	12·40	3·40	9·95
Biceps	4·60	28·50	4·48	32·00	4·50	28·32
Brachio-radialis	24·10	7·00	25·02	7·30	20·92	8·57
Hand-grip	32·0		32·0		—	—
Wristlet	25·4		25·2		—	—

X-ray photographs were taken of the arms of subjects M.R. and D.W., held in the experimental position and with the tube at least 5 ft. from the plate. The origins and insertions of the five flexors were identified from the photographs quite easily by comparing them with a skeleton of nearly the same size. The distances of these points from the joint axis were then measured (Table 3). In both subjects the angle BOE between humerus and horizontal was about 5°. The lever ratio at different angles of flexion was then calculated. The result is shown in Fig. 10.

It is clear that the lever ratio remains practically constant over the range of movement used experimentally, the only departure (radialis longus) being by extensor carpi.

The force-velocity relationship in individual muscles. Some uncertainty remains about the force-velocity relationship in individual muscles, because in each muscle the velocity of shortening is proportional to the horizontal component of the velocity of the hand (by exactly the argument detailed above in the case of forces), while the horizontal force at the hand is the *sum* of a number of proportional components, one from each flexor.

To illustrate the effect of this, imagine that the hand moves with a certain velocity V'. The various flexor muscles shorten at speeds proportional to V', and the tension which each one exerts is some function, probably the characteristic function, of the speed of shortening.

As V' increases, the rate of shortening of one of the muscles may eventually exceed its maximum unloaded speed V_0, so it will no longer be able to develop

tension. That is, it will drop out of action while the other flexors are still functioning. This is bound to happen unless in each muscle V_0 is proportional to OA (Fig. 9), the distance from insertion to joint axis. In the intact subject it is not possible to determine V_0 separately on each muscle; but if one assumes that the muscle fibres are similar in all the flexor muscles, V_0 should be proportional to the length of the muscle, P_0 to its cross-section. This assumes, of course, that the fibres all lie parallel with the long axis of the muscle. Examination of cadavers shows that this is in fact the case for all the flexors except brachialis.

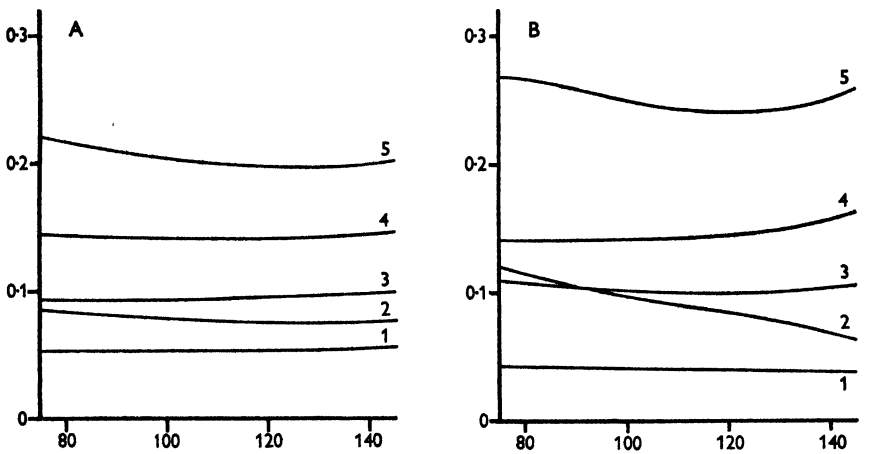


Fig. 10. Lever ratio at different angles of flexion. Abscissa: angle between forearm and horizon. Full extension is 180°. Ordinate: lever ratio $= \frac{\text{horizontal tension at the hand}}{\text{tension in muscle}}$. Curves: (1) Pronator teres; (2) extensor carpi radialis longus; (3) brachialis; (4) biceps, both heads taken together; (5) brachio-radialis. Graph A. Subject M.R. Graph B. Braune & Fischer (1890); mean value from four subjects.

The ratio (distance from axis OA/length of muscle) is certainly not constant, as it varies from 0·3 (brachialis) to 0·15 (biceps). If one knows the approximate dimensions of each muscle (Braune & Fischer, 1890, p. 291) it is quite a simple matter to calculate in arbitrary units the force developed by each muscle at various velocities of movement of the hand, for a given common value of a/P_0. The total tension is then the sum of the individual tensions, allowing for the difference in their lever arm. The resulting curve of total tension against velocity, e.g. Fig. 11, has discontinuities at the points at which brachialis and brachio-radialis drop out of action. Nevertheless, it can be fitted quite well by the characteristic equation with $a/P_0 = 0{\cdot}209$.

The value of a/P_0 for the combined curve is always small compared with that of the individual fibres, and it is difficult to explain how combined curves with a/P_0 as large as 0·48 (subject L.M.) could be built up in this way. It is likely,

therefore, that the intrinsic speed $(-dl/dt)/l$ under zero load, is not the same in the fibres of the different muscles. The same finding was reported in animals by Denny-Brown (1929), who demonstrated large differences between the speed of operation of different muscles in the same animal.

Even although there is some uncertainty about the exact *form* of the force-velocity relationship in the individual muscles, it is practically certain that each of them does follow a fixed force: velocity characteristic; for when the hand

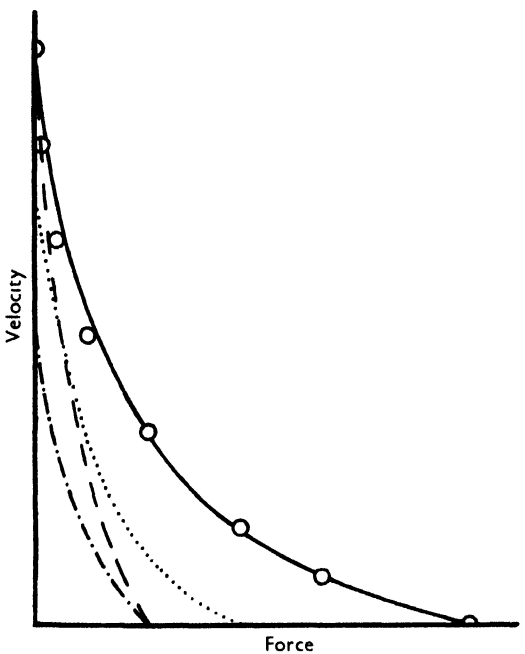


Fig. 11. Force-velocity relation in the three principal flexors of the elbow and in the whole limb. Values calculated on the assumption that the muscles are composed of similar fibres with $a/P_0 = 0{\cdot}4$. Forces and velocities expressed in arbitrary units as they would be measured at the hand. ....., brachialis; — — —, biceps; —.—.—.—, brachio-radialis; O—O—O— whole limb. Curve drawn from characteristic equation with $a/P_0 = 0{\cdot}209$.

moves at constant speed, each muscle shortens at constant speed, and the *total* tension remains constant. If the force exerted by each muscle does vary other than with the velocity, this variation must cancel out among the different flexors. It is very unlikely that this accident would be repeated in each of five subjects.

The tension-length diagram of human muscle. The isometric tension which a given muscle can exert varies with its length. The isometric tension, P_0, appears in the characteristic equation as a constant, so this equation can be expected to apply over only a limited range of shortening.

In order to ensure that this range was not being exceeded in these experiments, the variation of isometric tension in different positions of the limb was investigated.

The P_0 used in the equation is simply the horizontal tension exerted by the hand at zero speed of shortening and it is quite simple to measure this directly. It would be of considerable theoretical interest to discover what is the tension-length relationship in the individual flexor muscles. Unfortunately, the effect of one muscle cannot be isolated from that of the others; however, the number of active muscles can be cut down from five to two by anaesthetizing the radial nerve in the spiral groove. The resulting problem in interpretation is discussed below.

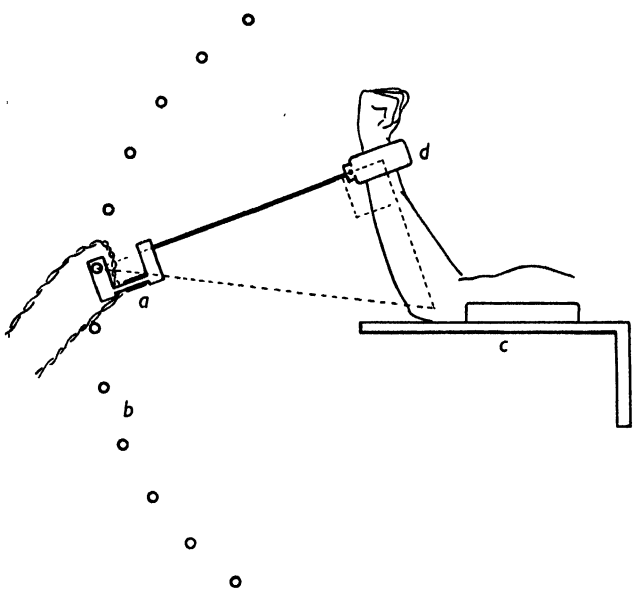


Fig. 12. Apparatus for measuring the isometric torque exerted in flexion of the elbow. *a*, tension gauge; *b*, circle of pegs mounted on wall; *c*, shelf to support arm, with plaster trough to prevent movement; *d*, plaster wristlet.

As it is impossible to grasp a hand-grip when the extensors of the wrist are paralysed, the horizontal force could not be measured directly. Instead, the torque exerted was measured by means of a plaster-and-metal wristlet attached by a cable to the tension gauge already described. The wristlet fitted snugly over the styloid processes of radius and ulna, and showed no tendency to slip either up or down. The gauge could be slipped on to one of a circle of pegs mounted on a wall. The length of cable was adjusted so that

$$\text{cable length} = \sqrt{[(\text{radius of circle of pegs})^2 - (\text{forearm length})^2]}.$$

Thus, by Pythagoras' Theorem, the angle between cable and forearm was always 90°.

In some experiments all the flexors, except biceps and brachialis, were paralysed by injecting a small amount of 4% procaine close to the radial nerve, about 12 cm. above the elbow. The resulting paralysis was complete for about an hour, and disappeared in 2–3 hr.

From the torque and the angle of the forearm, the horizontal component of the isometric force was calculated. A correction was made for the weight of limb and wristlet, which also exert some torque on the forearm. The variation in P_0 over the experimental range of movement (140–80°) is 13%, the tension increasing with increasing length of muscle. Most of the variation is in the first 20° of flexion, after which the isometric tension stays relatively constant.

To derive the tension-length diagram of individual muscles from measurements of the whole limb is a complicated problem. As the elbow flexes, the muscles alter in length at different rates; so at any particular position of the

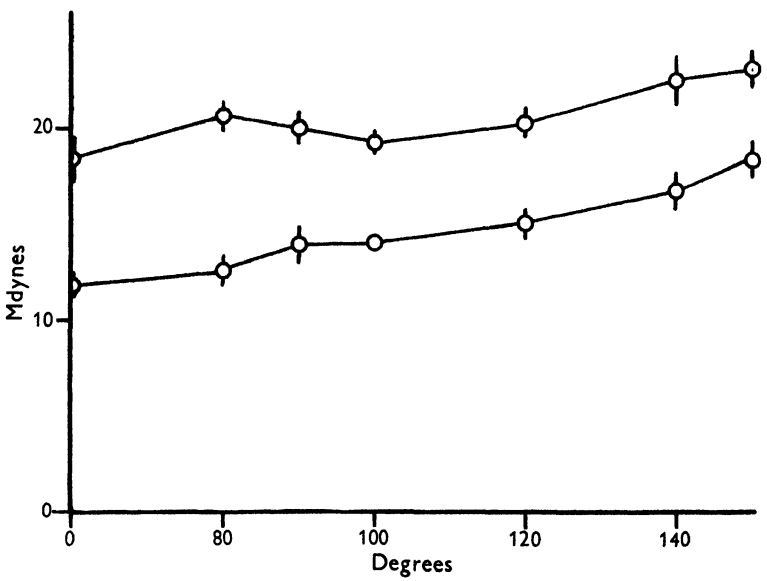


Fig. 13. Horizontal isometric tension produced at different angles of flexion. Subject M.R. Abscissa: angle between forearm and horizon. Complete extension = 180°. Ordinate: horizontal component of isometric tension, means of eight observations. Vertical bars: 6 × S.E. of mean. *A*, all flexors together; *B*, biceps and brachialis only.

limb they are almost certain to be on different parts of their tension-length curve. This makes it difficult to share the total tension between them on the basis of their relative cross-sections. Arbitrary assumptions on these points have been made by previous investigators (Franke, 1920; Hansen & Lindhard, 1923; Fenn, 1938). Reduction of the number of muscles involved simplifies analysis considerably, and the method attempted utilized only two assumptions: (1) that the percentile tension-length diagram of biceps was the same as that of brachialis, that is, that they were composed of similar fibres; (2) that the shape of their tension-length curve could be represented adequately by a four-term polynomial. Unfortunately, the patterns of shortening in the two muscles of the chosen subject were so similar that the resulting set of simul-

taneous equations, which were kindly investigated by Dr E. T. Goodwin of the National Physical Laboratory, proved to be malconditioned.

Ralston, Inman, Strait & Shaffrath (1947) have constructed tension-length diagrams obtained directly from measurements on the isolated muscles of human amputees. However, their results cannot be taken to represent the properties of *normal* human muscle, for their tension-length diagram of biceps (fig. 4, p. 615) shows a maximum change in length of only 0·9 in. (2·3 cm.), starting from the point at which no tension is developed. The shortening of biceps during a normal flexion is three times as great as this (Braune & Fischer, 1890), and is certainly not attended by so great a variation in tension; moreover, the maximum tension shown in their graph is less than one-fifth of that developed by a normal muscle.

The problem of excitation

In order to interpret experiments made on the whole limb it is necessary to decide whether the excitation resulting from maximal voluntary effort is constant throughout each movement, and whether it is the same in efforts made under different circumstances. The word 'excitation' is used to describe both the number of active units, and the degree of their activity, for the mechanical response is determined by both factors.

The constancy of the mechanical response at all times makes it very likely that the excitation does remain constant, but a direct investigation has also been made by recording electromyograms during the movements.

There are two distinct aspects to the investigation:

(A) To decide whether activity in the *agonist* muscles is constant in maximal efforts, no matter how other conditions change.

(B) To decide whether there is sufficient activity in *antagonist* muscles significantly to oppose the action of the agonists. If there is, and if this opposition varies, it may prove impossible to analyse the movement at all.

The quantitative interpretation of electromyograms

The action potentials picked up from a single motor unit during activity are all the same size and shape, mono-, di- or tri-phasic depending on the position of the electrode (Dirken & Siemelink, 1941; Denslow & Hasset, 1943; discussed theoretically by Pritchard, 1930). Increase in the activity of the motor unit is brought about by an increase in the frequency of discharge, the wave-form remaining constant. The Electromyogram (e.m.g.) is the record of potential changes picked up from many such motor units, between needles or skin electrodes some distance apart. This record is quite different from that of a single motor unit, for it consists of large irregular waves whose amplitude increases with increasing activity of the muscle. In spite of the irregularity there often appears to be a fundamental frequency of about 50 cyc./sec. This

rhythm has been considered to result from the synchronous discharge of anterior horn cells, though the amplitude of the e.m.g. at any instant depends as much on the phase-relations between contributing action potentials as on their frequency. Moreover, Dirken & Siemelink (1942) have shown that the peaks of the e.m.g. waves are not related in time to the action potentials of the underlying motor units. They suggest that the e.m.g. is determined statistically from the accidental differences between action potentials in different motor units.

Whatever the underlying mechanism may be, it has been demonstrated empirically (Haas, 1926; Inman, Saunders & Abbot, 1944) that in general terms, the amplitude of the waves increases as the weight supported in an isometric contraction increases. A variety of different measures of the amplitude have been used: mean height of waves (Hass, 1926); integral under curve (Ralston *et al.* 1947), and others; the number of waves greater than a certain size (Kennedy & Travis, 1947); or other combined parameters (Dempster & Finerty, 1947). As none of the measures has a theoretical foundation, the choice between them should be made on grounds of convenience.

One limitation intrinsic to all such measurements is that, as the waves vary considerably in size, the precision of estimation of *any* collective parameter (mean height, integral, etc.) must fall off rapidly as the length of record available diminishes. This limits the value of such measurements in quick movements, where in any case, no relationship between mechanical and electrical activity has been demonstrated. Only the most general quantitative interpretation is put on the electromyograms described below.

Reciprocal innervation

The role of antagonists in voluntary movement has been hotly disputed in the past. Some authors have maintained that anatomically antagonistic muscles showed reciprocal innervation; others that both groups always contracted simultaneously.

Apart from the technical difficulty, that a passive muscle may show potential changes conducted from an active neighbour, much confusion seems to have arisen from failure to define the mechanical conditions in the movement that has been studied.

Speaking very broadly, there are two types of voluntary movement:

(1) Movements in which only small external forces are involved, for example, relatively slow movements of the unloaded limb. In such movements precision and rigidity are increased by simultaneous contraction of agonists and antagonists; for in this way the sensitivity to small deflecting forces is decreased. This view is supported by the experimental findings of Brücke (1877), Rieger (1882), Tilney & Pike (1925), on precise movements; and of Wacholder & Altenburger (1926*b*) on voluntary rigidification.

(2) Movements against opposed external forces, or quick movements in which reaction provides an opposing force, are accompanied by relaxation of antagonists. This finding has been reported by Demeney (1890), Beevor (1891), Wacholder & Altenburger (1926*a*, *b*) and Hoefer (1941).

The experiments of Golla & Hetwer (1924, pp. 62–63) illustrate clearly the difference to be expected in the two types of movement. In those of Hathaway (1935) the mechanical conditions are not defined, as they had no relation to the object of the research, but one record (fig. 2, p. 292) shows only slight antagonistic activity in a moderately fast extension of the unweighted forearm.

Animal experiments have increased confusion, for cortical stimulation can give rise to either type of movement, resulting in reciprocal innervation (Sherrington, 1894), or co-contraction (Tilney & Pike, 1925). This difference has been shown to depend on the strength of stimulation (Bosma & Gellhorn, 1946).

METHODS

It appeared that no electromyographic experiments had been performed under precisely the same conditions as the mechanical experiments reported above. Recordings were accordingly made from biceps and triceps during maximal voluntary flexions.

Amplifiers. Two independent capacity-coupled amplifiers connected to two pens of a Hughes four-channel recorder, recording on dry electrolytic paper. Overall frequency response maximal at 100 c.p.s., 3 db. down at 40 and 250 c.p.s.

Displacement indicator. The axle of the isotonic lever was attached to the contact arm of a sine-cosine potentiometer, arranged to provide a voltage proportional to the horizontal component of the displacement of the hand. This signal deflected the third pen of the recorder, while the fourth channel was used to register time marks every 50 msec.

Electrodes. Originally skin electrodes were used. They were made by soldering thin flex to 4 B.A. washers, the component wires being splayed out so as to cover the hole and leave a hollow for electrode jelly.

With these electrodes localization was poor: even in isometric flexions, in which triceps was palpably relaxed, potentials were picked up by conduction from biceps. By using needle electrodes localization was certainly improved, but the volume of muscle in which activity could be detected was very small.

The compromise finally adopted was to use bare hypodermic needles 2 cm. apart in triceps, retaining skin electrodes 2 cm. apart over biceps. It is essential that the electrodes should cause no discomfort, for otherwise it is quite impossible for the subject to make an unguarded maximal effort. This condition was secured by using very sharp needles inserted about 10 and 12 cm. below the origin of the muscle. In this region there is only slight movement between skin and underlying muscle, and with luck positions can be found in which the needles are quite imperceptible.

Mechanical arrangements. The isotonic lever was arranged in exactly the same way as already described, except that the weights were not held up at the end of the movement by the catch.

RESULTS

The result of a typical experiment is shown in Fig. 14. Two facts are demonstrated in these records:

(1) Action potentials appear in biceps some time before any movement is apparent, as described by Hathaway (1935). So far as one can judge from the

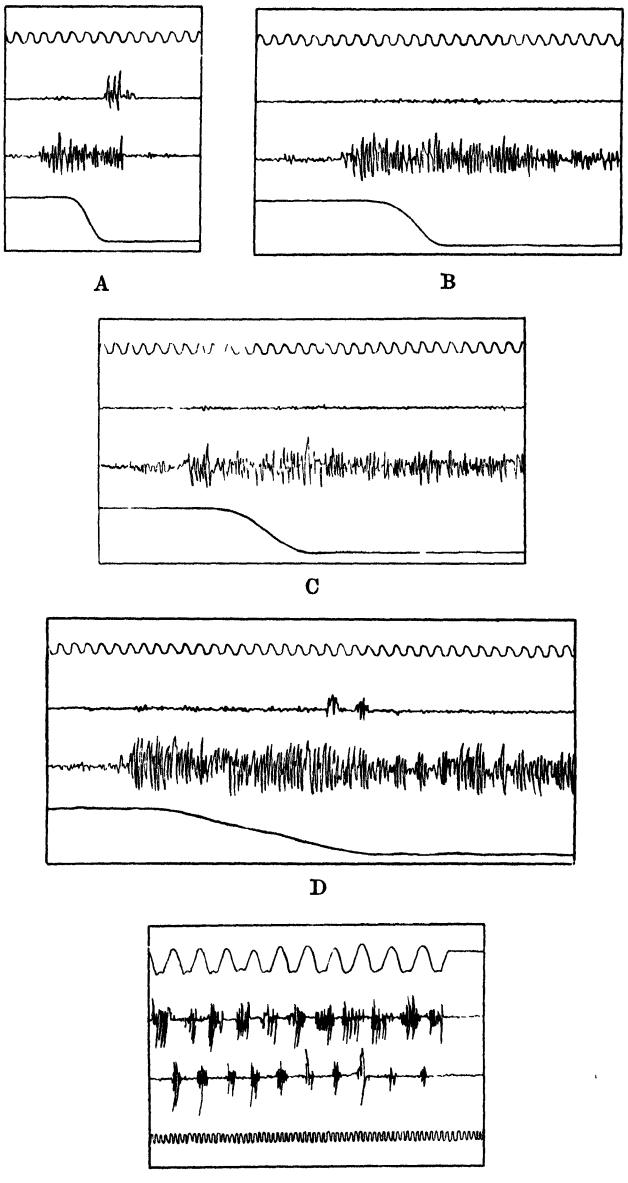


Fig. 14. Electromyograms showing reciprocal innervation. Four channels from above downwards. Time, 20 cyc./sec. Amplifier: needle electrode in triceps. Amplifier: skin electrodes over biceps. Displacement indicator: downward deflexion of trace proportional to horizontal component of hand movement during flexion. Maximal efforts. Subject J.H. A. Inertia of arm and lever only. B. Additional isotonic force of 2·47 megadynes, held up by subject at end of movement. C. Isotonic force 5·40 megadynes, held up. D. Isotonic force 7·0 megadynes, held up. E. Rapid to-and-fro movement of forearm.

amplitude of the record, activity has reached its maximum before movement begins: it also appears that the degree of activity is practically the same under different conditions of loading.

(2) Activity in the antagonist appears only in records A and D. In record A the function of triceps is clearly to check the rapid movement of the limb. This is no longer necessary when there is an opposing isotonic force. The significance of the twin outburst of activity in D is not clear. It occurs just at the end of the movement, so it may be that the limb is rigidified in preparation for the heavy load it must support.

DISCUSSION

An independent investigation of the same problem has been made recently by Dern *et al.* (1947), and I should like to take this opportunity to thank Dr R. Dern for his kindness in allowing me to see the typescript of their paper, as well as his own Ph.D. thesis on the same subject.

Most of their experiments were made in angular measure (torque, angular velocity, moment of inertia), instead of linear measure; so it is difficult to interpret them in terms of muscle function. Their general conclusion is that a constant torque is produced by the flexor muscles over a considerable part of the movement. This is not necessarily inconsistent with the theory put forward in this paper. When velocity-time curves (Fig. 5A, p. 259, 4·53, 13·42 megadynes) were transposed to circular measure, it was found that the torque exerted by the muscles did remain constant, but for only 100 and 50 msec. respectively in the two cases.

Moreover, in experiments in which Dern *et al.* applied horizontal isotonic forces, they obtained hyperbolic force-velocity curves. The high values obtained for a/P_0 (0·43–0·63) are to be expected if no correction is made for inertia.

Thus the only major difference in experimental findings is that Dern *et al.* find sufficient activity in antagonist muscles throughout movement to prevent any interpretation in terms of the function of individual muscles. This does not appear to be the case in the present experiments, nor would it be expected from the observations of other investigators (see p. 273).

Hill's equation is a very convenient one for describing force-velocity curves, and all those encountered so far have been easily fitted. No attempt is made to identify it with any particular mechanism of contraction, for, depending on the number of mathematical constants allowed, many other equations could be devised to fit as well. The adoption of any other equation would not affect the predictions made of the force-velocity and tension-time curves, since these are based strictly on the two hypotheses:

(1) That the velocity of maximal contraction depends only on the tension. (No particular mathematical function is specified.)

(2) That there is an elastic element in series with the contractile one.

Comparison of human with other muscle. In determining the relationship between force and velocity in human muscle, apart from skeletal and nervous complications, most of the difficulties have been associated with the high inertia of the forearm, which made an elaborate mechanical analysis necessary.

In work on isolated frog muscle these difficulties may be largely avoided by working at 0° C., when the speed of contraction is considerably reduced, and by arranging that the inertia of the lever, unlike that of the forearm, is 'geared down' to the muscle. In addition, the intrinsic properties of the muscle make it less sensitive to inertia. The values of P_0 and V_0 are approximately 20 kg. wt., 700 cm./sec. for a man; 50 g. wt. and 5 cm./sec. for a frog sartorius at 0° C. To have the same effect on both muscles the inertias required will be in the ratio 20/0·7:50/5, or about 3:1. That is, a sartorius lever must have an equivalent mass of 200 g. to have the same delaying effect as the forearm.

Optimal conditions for doing work. The rate of doing work is zero when $P=0$ or $P=P_0$, and maximal when

$$\frac{P}{a}=\sqrt{\left(1+\frac{P_0}{a}\right)}-1 \quad \text{(Hill, 1938).}$$

During the movement of flexion of the elbow the maximal rate of working attained is surprisingly high: in the case of subject M.R., for example, when $P=7$ megadynes, $V=200$ cm./sec.; the power output is then 140 W., or one-fifth of a horse-power.

Many everyday appliances are designed so as to enable a given task to be performed at the optimal rate of working. It is the necessity for matching that determines the gearing of bicycles, for example, or the size of hand implements. Detailed knowledge of muscle physiology cannot yet be substituted for practical judgement in the design of such appliances: except possibly in the case of surgical prostheses they involve movements too complex for mathematical analysis. However, the principle involved must be recognized by designers. Fast movement cannot be achieved in the presence of heavy loading: a subject can best perform a mechanical task if it is matched to the properties of his muscles.

SUMMARY

1. The relationship between isotonic force (P) and velocity of movement (V) has been studied in maximal flexions of the elbow.

2. After correction for the inertia of the forearm the $P:V$ curve can be represented by Hill's equation

$$(P+a)\,(V+b)=(P_0+a)\,b,$$

where P_0 is the isometric tension, a and b are constants. a/P_0 varied from 0·20 to 0·48 in five subjects.

3. The hypothesis that there is an inert elastic element in muscle makes it possible to relate experimental isotonic and isometric contractions to the $P:V$ curve.

4. The problem of deriving muscle properties from measurements on the whole limb is examined. The geometry of the flexor muscles and their tension-length diagram are investigated, and it is shown to be unlikely that all the muscle fibres involved have identical properties.

5. The quantitative interpretation of electromyograms is discussed. Antagonist activity is shown to be absent during experimental movements.

I wish to express my gratitude to Prof. A. V. Hill for his kindly help and criticism; also to thank Mr J. L. Parkinson for much practical assistance, including the construction of the isotonic lever, and Mr A. C. Downing for the loan of galvanometers. Other members of the Department have helped me considerably by their advice and by acting as subjects.

The latter part of this research has been aided by a grant from the Royal Society.

APPENDIX A

A method of correcting for inertia

Owing to the limited range of movement of the elbow joint, and to its own inertia, the forearm does not have time to reach its full velocity. If it is assumed that the muscle obeys the characteristic equation the size of the velocity deficit can be estimated for a given length of pull. The method of calculation is due to Prof. A. V. Hill. On integrating equation 1 (p. 254),

$$t=-\frac{MV}{(a+F)}-\frac{Mb(P_0+a)}{(F+a)^2}\log\left(1-\frac{V(F+a)}{b(P_0-F)}\right).$$

This equation describes how the velocity rises with time during a single contraction. The theoretical final velocity at $t=\infty$, which is the same as the velocity disregarding inertia, is $V_1=\frac{b(P_0-F)}{F+a}$ from the characteristic equation. Inserting this above, putting

$$\alpha=\frac{P_0-F}{P_0+a},$$

and

$$\beta=\frac{(F+a)^2}{(P_0+a)\,Mb},$$

then

$$\beta t=-\log\left(1-\frac{V}{V_1}\right)-\alpha\frac{V}{V_1}.$$

Differentiate with respect to t and rearrange, putting $dV/dt=dV/dx\times dx/dt$, where x is displacement,

$$\beta=\frac{dV}{dx}-\alpha\frac{V}{V_1}+\frac{V_1}{V_1-V}-1.$$

Integrate, putting $x=0$ at $V=0$,

$$\beta \frac{x}{V_1} = -\log\left(1-\frac{V}{V_1}\right) - \frac{V}{V_1} - \tfrac{1}{2}\alpha \frac{V^2}{V_1^2}.$$

This equation describes how the velocity rises with the distance pulled. As V/V_1 is very nearly unity in practice, this equation can be simplified. Put $V/V_1 = 1-Z$, when terms in Z and Z^2 can be neglected in comparison with $-\log Z$. Then

$$\beta x/V_1 = -(\log Z + 1 + \alpha/2),$$

which is the expression required. V_1, α, β, and x are known from the provisional characteristic equation, the force F and the measured length of pull. Z, and from it V/V_1, can therefore be calculated. The experimental values of velocity are then divided by V/V_1 to obtain an estimate of the theoretical final velocity.

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GLUCOSE ABSORPTION FROM SURVIVING RAT SMALL INTESTINE

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As a first test of the utility of the surviving intestine preparation recently described (Fisher & Parsons, 1949), a study of active glucose absorption was undertaken. The work has dealt with several points of general interest:

(1) Since, using the surviving preparation, the glucose concentration to which a segment is exposed can be specified reasonably precisely, it has been possible to study quantitatively gradients of activity in the intestine.

(2) Since it is also possible to obtain estimates of glucose utilization of intestinal segments in the closed system of the surviving preparation, the process of absorption from the intestinal lumen can be separated into component processes of utilization and translocation of glucose.

(3) Studies under these two heads provide a basis for computing the capacity of the whole small intestine to absorb glucose at a specified concentration: such an estimate throws light on the interpretation to be placed on absorption studies carried out on the whole animal.

METHODS

The setting up of the intestinal preparation has already been described (Fisher & Parsons, 1949).

In view of the relative simplicity of the fluids to be analysed, total reducing substances were determined by the Hagedorn-Jensen (1923) method, after deproteinizing with $Zn(OH)_2$. In experiments with high glucose concentrations Hulme & Narain's (1931) modification of Hanes's (1929) modification of the Hagedorn-Jensen method was used.

RESULTS

Active absorption of glucose. A series of experiments was made in which the inner and outer fluids initially contained the same concentration of glucose, in the region of 450–500 mg./100 ml. In the course of an hour a fall in inner fluid glucose concentration and a rise in outer fluid glucose concentration were usually observed. Table 1 shows representative figures which are in themselves sufficient evidence for active translocation, either of glucose in the one direction or of water in the other.

TABLE 1. Glucose concentration in inner and outer fluids in experiments in which segments intestine are maintained for 1 hr. periods on circulation units. J = jejunal segment, I = ileal segment

Exp.	Segment position	Segment length (cm.)	Glucose concentrations in circuits (mg./100 ml.) Inner Initial	Inner Final	Outer Initial	Outer Final	Reduction in inner fluid vol. (%)
1	J	49·0	416	228	459	502	18
2	J	45·0	381	266	427	481	20
3	J	37·0	429	288	466	499	22
4	J	62·5	418	250	438	490	26
5	I	49·5	438	345	455	493	11
6	I	48·5	434	401	467	452	16
7	I	49·0	440	302	438	466	14
8	I	47·0	448	357	448	438	14

The last column of Table 1 gives the percentage decrease in inner fluid volume during the hour, determined by the methods already described (Fisher & Parsons, 1949), and makes it clear that, although movement of water does occur, it is in the direction opposite to that necessary to account for the changes in glucose concentration. When the data are computed in terms of glucose content of the two circuits, allowance thus being made for the water shift, the figures of Table 2 are obtained. There is no doubt here that active translocation of glucose occurs, and that the extent of the translocation is very different in different segments.

TABLE 2. Total glucose contents of inner and outer fluids in the experiments listed in Table 1

Exp.	Segment position	Total glucose (mg.) Inner circuit Initial	Inner circuit Final	Inner circuit Diff.	Outer circuit Initial	Outer circuit Final	Outer circuit Diff.
1	J	222	124	−98	221	291	+70
2	J	217	125	−92	216	292	+76
3	J	231	123	−108	230	269	+39
4	J	232	107	−125	232	272	+40
5	I	221	157	−64	221	253	+32
6	I	222	173	−49	221	258	+37
7	I	231	133	−98	231	257	+26
8	I	224	158	−66	224	246	+22

Quantitative specification of absorptive activity. As our animals have exhibited a relatively narrow range of body weights (200–300 g.) the question of relating absorption to body weight or surface area is an academic one. Further, we have seen no relation between body weight and length of intestine, in agreement with Westenbrink's (1936) finding of absence of correlation between body weight and intestinal weight. The simplest measure of absorption available is quantity absorbed per unit serosal length of intestine, and this has proved satisfactory. The results in this paper are expressed throughout in mg./cm./hr. signifying mg. of glucose absorbed from the lumen, translocated across the

mucosa, or utilized, as the case may be, per cm. serosal length of intestine per hr. period of maintenance on the circulation unit. The length of the intestine was measured in these experiments by laying it out without tension on a metre rule at the end of the survival period.

Table 3 gives the results of a series of determinations expressed in these units, together with the simplest available measure of location of the segments, the mean distance of the segment from the ileo-caecal valve (ICV). The mean distance is the distance of the midpoint of the segment from the ICV. It is clear from this table that there is a regular relation between absorptive activity measured in mg./cm./hr. and location measured as mean distance.

TABLE 3. Relation between mean distance of segment from the ileo-caecal valve and the rate of disappearance of glucose from the inner fluid in all available experiments of the type listed in Tables 1 and 2. For explanation of third column see the text

Mean distance (cm.)	Disappearance of glucose (mg./cm./hr.) individual observations	Standardized absorptive activity (mg./cm./hr.)
76	2·36	1·32
73	2·68	1·73
72	2·89	1·97
69	2·21	1·38
63	2·50	1·86
63	2·07	1·43
	Group mean 2·45	1·62
56	2·05	1·63
51	2·06	1·80
50	1·71	1·48
42	1·68	1·70
40	1·47	1·56
	Group mean 1·79	1·63
29	0·94	1·37
26	1·10	1·62
26	0·87	1·39
24	1·40	1·99
24	1·02	1·61
23	1·38	2·00
23	1·25	1·87
21	1·09	1·77
19	0·75	1·49
	Group mean 1·09	1·68

The gradient of glucose absorption. Fig. 1 shows a plot of absorptive activity in mg./cm./hr. against mean distance from ICV in cm. for all available data, including those of Table 3. Although the points represent data collected from seventeen different animals they lie well around a straight line, and there is no doubt of a systematic linear relation between absorptive activity and mean distance from the ICV. We refer in this paper to the equation describing this and similar relations as 'gradients' of intestinal activity.

As an aid to the physical interpretation of a gradient expressed in these terms it may be pointed out that a linear dependence of absorptive activity on mean

distance implies that the average absorption per cm. of intestine for any segment whose mean distance from the ICV is m, is equal to the rate of absorption from the centimetre length of intestine whose midpoint is at a distance m from the ICV. The magnitude of the gradient is striking, the mean absorptive activity of a cm. length at 80 cm. from the ICV (corresponding to the upper end

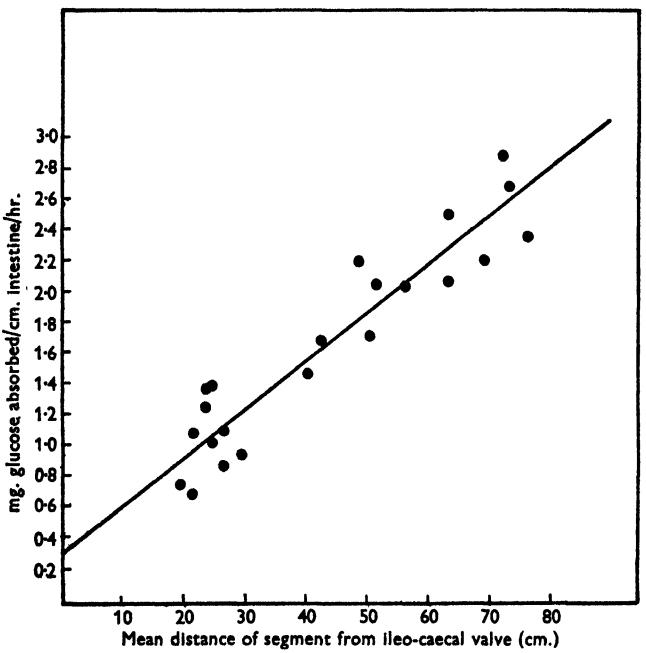

Fig. 1. Relation between glucose disappearance from inner fluid and mean distance of segment from ileo-caecal valve in 1 hr. experiments with initial concentrations of 0·5% glucose in inner and outer fluids.

of the jejunum) being over nine times that to be expected for a cm. length at the ICV. The regression line relating absorptive activity, A, to mean distance, m, which is shown in Fig. 1 has the equation

$$A = 0{\cdot}0313m + 0{\cdot}317. \tag{1}$$

We have tested statistically the possibilities that closer relations between absorptive activity and mean distance might be obtained by taking into account variations in total length of small intestine or in body weight or in both, but have found only trivial effects of these variables on the goodness of fit of the data to a linear relation between absorptive activity and mean distance. The relation expressed in the equation above has therefore been used to provide directly a means of computing a 'standardized absorptive activity' which makes it possible to compare the activities of segments from different locations.

Standardized absorptive activity (SAA). If the equation of the linear regression of absorptive activity on mean distance is

$$A = bm + c,$$

where A is absorptive activity in mg./cm./hr. and m is mean distance in cm., and b and c are constants, and if M is the average value of mean distance for the set of segments to be compared, then the SAA of a segment whose mean distance is m_j and whose measured absorptive activity is a_j is given by

$$(\text{SAA})_j = a_j + b(M - m_j). \tag{2}$$

When this method is applied to the data of the first and second columns of Table 3 the values given in the third column are obtained. The data of this table are arranged in descending order of mean distance, and group means for the observed absorptive activity and the standardized absorptive activity are given for the three mean distance ranges 19–29, 40–56 and 63–76 cm. It will be seen that the group mean standardized absorptive activities are very similar in these three ranges, although the observed activities differ largely.

Gradient of glucose utilization. It appeared likely that extension of the earlier work (Fisher & Parsons, 1949) on utilization of glucose in conditions comparable to those of the experiments just described might aid in the interpretation of the gradient of glucose absorption. Further experiments similar to those in Table 3 of the previous paper were therefore made. Using 0·5% glucose in inner and outer fluids, nine pairs of segments were maintained on circulation units for 60 min., the total sugar in fluids and intestine being then determined in the way previously described. A further seven pairs of segments were maintained on circulation units for 5 min. only. The differences between glucose found at the end of these periods and glucose introduced in inner and outer fluids are plotted in Fig. 2 against the mean distance of the segments from the ICV. (In computing mean distance in these experiments, allowance has been made for the contraction of the intestine which occurs when it is plunged in boiling water to arrest glycolysis.)

In the figure pairs of points corresponding to segments from the same animal are joined by straight lines. These data suggest that the rate of change of glucose utilization from point to point along the intestine is of much the same order in the different animals. On the other hand, the mean apparent glucose utilization differs widely from animal to animal. This is probably because there is a wide variation in amount of pre-formed reducing substance in the intestine, allowance for which cannot be made directly. The data for the 5 min. experiments support this suggestion. In general, more glucose is recovered at the end of the 5 min. experiments than was present initially in the Ringer solutions, and the scatter of the mean apparent glucose appearances in this series is much the same as the scatter of the mean apparent glucose disappearances in the 60 min. experiments.

It would have been preferable to have found some means of preparing animals so that the content of the pre-formed reducing substance in the intestine was sensibly constant, had there been any clue to the factors involved. In the absence of any such clue, the data of Fig. 2 were used to compute an approximate corrected gradient of glucose utilization. The regressions of apparent

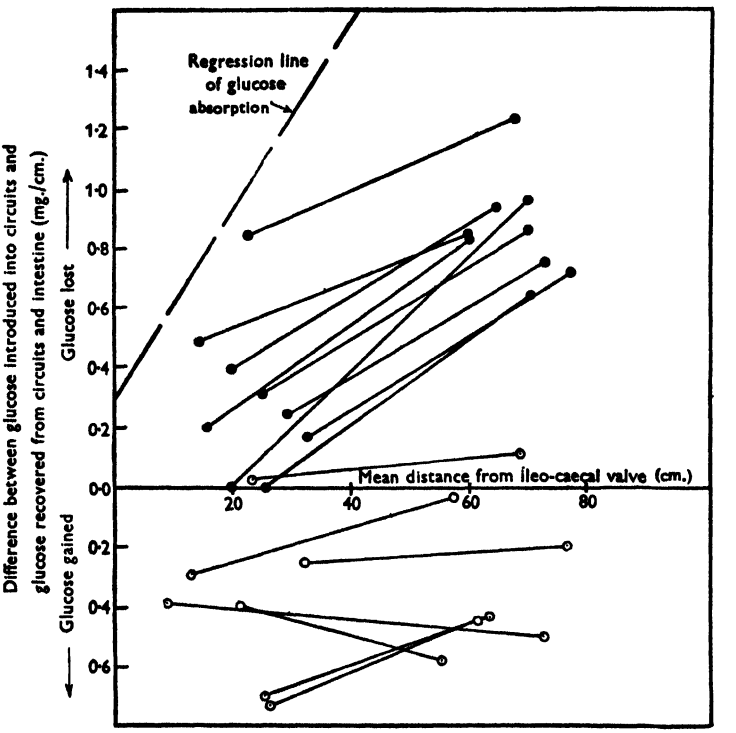

Fig. 2. Difference between glucose introduced in circulating fluids and glucose recovered from circulating fluids and intestine in 60 min. and 5 min. experiments with initial concentrations of 0·5% glucose in inner and outer fluids. o—o = Utilization of glucose 5 min. •—• = Utilization of glucose 60 min. Each pair of points joined by the line represents results from one animal.

change in glucose content of the system on mean distance were computed for the 60 min. and for the 5 min. series. Signifying apparent disappearance of glucose by ΔG mg./cm. and mean distance by m cm. the equations of these regressions are:

60 *min.* $$\Delta G = 0{\cdot}0109m + 0{\cdot}093, \quad (3)$$

5 *min.* $$\Delta G = 0{\cdot}0021m - 0{\cdot}429. \quad (4)$$

Subtracting the second of these equations from the first should give the gradient

of glucose utilization per 55 min., corrected for variations in pre-formed reducing substance. The equation so obtained is

55 *min. utilization* (*corrected*)

$$\Delta G = 0{\cdot}0088m + 0{\cdot}521, \tag{5}$$

and this, when multiplied through by 60/55, gives the estimated gradient of glucose utilization in mg./cm./hr. which is

$$U = 0{\cdot}0096m + 0{\cdot}568. \tag{6}$$

Comparison of (6) with (3) indicates that the correction introduced by allowing for the 5 min. results is almost equivalent to the addition of a constant allowance for pre-formed reducing substance. As a result of the wide variation of this term from individual to individual, the constant term of (6) is not fixed with any great precision. On the other hand, it can be shown that the value obtained for the slope of (6) is significantly less than the slope of the glucose absorption gradient (equation (1) and Fig. 1). The line representing the glucose absorption gradient has been reproduced also on Fig. 2 to provide a direct comparison.

Gradient of glucose translocation. We have now established that an appreciable fraction of the glucose disappearing from the inner fluid is utilized by the intestine and that the gradient of this utilization is markedly less than the gradient of absorption. This implies that the gradient of the second process involved in disappearance of glucose from the inner fluid, namely glucose translocation across the mucosa, must also be markedly different from the glucose absorption gradient. The translocation gradient can be obtained directly, by a process analogous to that used in the previous section, by subtraction of equation (6) from equation (1), giving

$$T = A - U = 0{\cdot}0217m - 0{\cdot}251. \tag{7}$$

The negative constant term here would appear to imply that translocation occurs *into* the intestinal lumen in the lowest 10–15 cm. of small intestine. However, the value of this term is not fixed with any great precision, the standard error of the experimental estimate being almost exactly equal to the estimate itself. There is approximately a 1 in 6 chance that a repetition of this work would yield a positive constant term for the translocation gradient.

Effects of phlorrhizin on active glucose absorption. When 100 mg./100 ml. of phlorrhizin is present in both inner and outer fluids in experiments in which both fluids contain initially 0·5 % glucose, there is a large decrease in absorptive activity. Table 4 shows the relation between absorptive activity and location for six pairs of segments, one segment from each animal being exposed to phlorrhizin, the treatments being equally divided between ileal and jejunal segments. The regressions of absorptive activity on mean distance have been calculated separately for the phlorrhizin and the control series and used to

calculate SAA's referred to the overall average mean distance (43·7 cm. from ICV) for both series. These computations give an SAA in the phlorrhizin series of 0·40 ± 0·22 mg./cm./hr. compared with an SAA of 1·52 ± 0·33 mg./cm./hr. in the control series. Thus, in these conditions, phlorrhizin significantly depresses glucose disappearance from the inner fluid, and on average depresses it to 26 % of the control rate. Since the phlorrhizin rate observed here is considerably lower than the rates of either translocation or utilization to be expected at 43·7 cm. from the ICV (equations (6) and (7) give 0·99 and 0·69 mg./cm./hr. for utilization and translocation respectively at this distance), it is reasonable to suppose that both utilization and translocation are depressed by phlorrhizin.

TABLE 4. Effect of phlorrhizin (100 mg./100 ml.) on rate of disappearance of glucose (ΔG in mg./cm./hr.) from inner fluids. Third and sixth columns give standardized absorptive activities (SAA in mg./cm./hr.) referred to the average mean distance of all twelve segments listed (43·7 cm. from ICV)

Phlorrhizin			Control		
Mean distance (cm.)	ΔG	SAA	Mean distance (cm.)	ΔG	SAA
65	0·50	0·61	56	2·05	1·59
60	0·10	0·19	72	2·89	1·83
79	0·23	0·41	71	2·00	0·97
17	0·33	0·21	22	0·69	1·50
27	0·39	0·31	19	0·75	1·68
10	0·83	0·66	26	0·87	1·54

One other interesting aspect of the phlorrhizin effect is illustrated by Table 5, in which inner and outer glucose concentrations at the end of the hour survival period, and shifts of water out of the inner fluid are set out as in Table 1. The water shifts in the presence of phlorrhizin are smaller than in Table 1, but the indication is that in the presence of phlorrhizin water leaves the inner circuit more rapidly than glucose, since the final outer circuit glucose concentrations are uniformly lower than the initial concentrations. This effect cannot readily be interpreted in terms of filtration, and would appear to indicate an active translocation of water less sensitive to phlorrhizin than is glucose translocation.

TABLE 5. Glucose concentrations in inner and outer fluids in experiments in which segments of intestine are maintained for 1 hr. in circulation units, with 100 mg./100 ml. phlorrhizin in both fluids

Segment position	Segment length (cm.)	Glucose concentration in circuits (mg./100 ml.) inner Initial	inner Final	outer Initial	outer Final	Reduction in inner fluid vol. (%)
J	47·0	434	444	474	442	13
J	38·5	490	486	501	490	7
J	43·5	441	454	441	418	5
J	54·5	493	502	521	484	7
I	33·0	415	427	492	462	−5
I	53·0	489	491	502	490	9
I	19·5	479	495	489	458	10

Retrograde movement of glucose. Further experiments were made with the inner fluid free from glucose, the outer fluid containing the usual 0·5 % glucose. In these experiments there is regularly a disappearance from the outer fluid of the same order as that to be expected from the utilization figures, but the amounts of glucose appearing in the inner fluids have been within the limits of

TABLE 6. Effect of phlorrhizin (100 mg./100 ml.) on disappearance of glucose from outer fluid and appearance of glucose in inner fluid in one hour maintenance experiments in which glucose (0·5 %) is added to outer fluid only

Control (mg./cm./hr.)			Phlorrhizinized (mg./cm./hr.)		
Segment position	Appearance of glucose in inner fluid	Disappearance of glucose from outer fluid	Segment position	Appearance of glucose in inner fluid	Disappearance of glucose from outer fluid
I	0·018	0·772	J	0·282	0·631
J	0·005	0·941	I	0·350	0·247
I	0·074	0·490	J	0·222	0·367
I	0·003	0·441	J	0·187	0·232
J	0·004	0·691	I	0·273	0·260
I	0·004	0·572	J	0·302	0·605
Weighted means	0·015	0·692		0·280	0·356

Weighted means are here calculated to give equal weight to the average values of the series of ileal and jejunal segments available, e.g. in the second column the mean of the four ileal segment figures is 0·0248, that of the two jejunal segment figures is 0·0045, and the average of these two values is the weighted mean given at the foot of the column.

error of the analytical method. Parallel experiments with 100 mg./100 ml. phlorrhizin have shown uniformly lower rates of glucose disappearance from the outer fluid than in the unpoisoned segments, together with significant appearances of glucose in the inner fluid. The results of these experiments are given in Table 6. Two points are of interest here. The figure for the unpoisoned segments (0·69 mg./cm./hr.) is rather less than the mean utilization found when glucose is present in both inner and outer fluids and must be interpreted as a utilization since there is no translocation. Since in the present experiments only one pole of the mucosal cells is exposed to glucose, and the utilization rate might therefore reasonably be expected to be lower than when both poles are exposed to the same glucose concentration, these figures may be taken as evidence that there is no substantial barrier to diffusion of glucose across the submucosal tissues. The second point is that in the phlorrhizinized segments the weighted mean figure for appearance of glucose in the inner fluid (0·280 mg./cm./hr.) is little less than the weighted mean estimate of disappearance of glucose from the outer fluid (0·356 mg./cm./hr.). Thus the phlorrhizin must be taken to have reduced both glucose utilization and translocation to small proportions. Even in the absence of utilization or translocation of glucose some difference between glucose disappearance from the outer fluid and glucose appearance in the inner fluid is to be expected, since glucose will be retained in the intestinal wall in amount sufficient to come into diffusion equilibrium with that fraction of the tissue water accessible to it.

Experiments with high glucose concentrations. Attempts to study active absorption at high glucose concentrations have given unsatisfactory results. At 2·5% glucose in both fluids, disappearance of glucose from the inner fluid is observed, but no appearance in the outer fluid. This may mean that the mucosal cells cannot concentrate glucose to a higher level than 2·5%, or that they are damaged by exposure to high glucose concentration in the outer fluid. In other experiments, with 2·5% glucose in the inner fluid only no difference in rate of disappearance of glucose from the inner fluid was produced by phlorrhinzin (100 mg./100 ml.). This would be expected if phlorrhizin poisoning were a competitive inhibition, but is unfortunate since it makes it impossible to estimate the rate of active translocation at high glucose concentrations.

The experiments with 2·5% glucose in both fluids, in which no translocation occurred, provide a rough estimate of glucose utilization at this concentration, the figure being 2·5 mg./cm./hr., as compared with 1 mg./cm./hr. at 0·5% glucose concentration. In the experiments with glucose in the inner circuit only, the rate of passage into the outer fluid averages 2·5 mg./cm./hr. as against an active translocation of 1·5 mg./cm./hr. at 0·5% glucose concentration. Active translocation at the higher concentration is therefore not likely to be more than one and a half times as great as at the lower.

DISCUSSION

The movement of glucose across the intestinal mucosa against a concentration gradient appears to have been demonstrated only once previously in experiments by Bárány & Sperber (1939) on anaesthetized rabbits. In these experiments mixtures of glucose with sorbose or with sodium sulphate which were isotonic with blood were introduced into tied-off segments of small intestine. After 2 hr. the residual fluid in the lumen frequently contained glucose in lower concentration than in the blood.

The demonstration of a gradient of absorption of glucose from the intestinal lumen is in agreement with much earlier work, though the position has not previously been made clear (see, for example, Verzár & McDougall, 1936, p. 113). The earlier work consisted exclusively of comparisons of activity of different intestinal segments in which no quantitative account was taken of location of the segments. This may account for the frequent contradictory results. As Fig. 1 demonstrates, when segments are ranked quantitatively in respect to location, there is no doubt of the reality of the gradient.

Another new feature of the present work is the separation of the gradient of absorption from the lumen into component gradients of utilization and translocation, 'utilization' covering all processes which convert glucose into forms not estimated by the analytical method used, and 'translocation' signifying the resultant of all processes tending to transfer glucose from one side of the mucosa to the other. The ability to specify these component gradients quanti-

tatively which is conferred by the technique used makes it possible to compare their characters with each other and with other intestinal gradients.

The one other intestinal gradient which is susceptible to simple quantitative specification is that of mucosal area per unit serosal length (MA/SL). Verzár & McDougall's (1936, p. 10) data for the pigeon agree with those of Warren (1939) for the dog in suggesting that, when the duodenum is excluded, there is a linear MA/SL gradient. The data for the rat are less full (Wood, 1944) and do not in themselves establish the form of the MA/SL gradient in rat intestine. They serve to show, however, that MA/SL is appreciably greater in the jejunum than in the ileum. Limits to the form of an assumed linear gradient can be derived from these published data by making extreme assumptions about the locations of the microscopical preparations measured.

It is possible to test the possibility that two gradients may be related. If, say, absorptive activity per unit serosal length, A, is proportional to mucosal area per unit serosal length, S, at any location in the intestine, so that $A = kS$, then the linear gradient $S = bm + c$ implies that $A = kbm + kc$. That is, the ratio of the slope (b or kb) to the intercept (c or kc) is the same for the two gradients.

The two directly determined gradients to be compared with the MA/SL gradient are those of absorption of glucose from the inner fluid and of glucose utilization. The slope/intercept ratios for these gradients are given below, together with that for the MA/SL gradient, the two values given for this last ratio being those corresponding to extreme assumptions concerning the location of the intestinal segments measured by Wood (1944).

Gradient	Slope/intercept ratio
Absorption of glucose from inner fluid	0·099
Glucose utilization	0·017
MA/SL	0·007–0·020

The utilization gradient ratio is within the range of estimates of the MA/SL gradient ratio, so that it is reasonable to suppose that utilization is proportional to mucosal surface area. On the other hand, the absorption gradient ratio is much higher. The 5% fiducial limits of this ratio, computed by the method given by R. A. Fisher (1948, p. 142) are 0·046–0·455, that is, wholly outside the likely range for the MA/SL ratio.

Since the absorption gradient is the sum of the utilization and translocation gradients, the finding that the absorption gradient must be taken to be significantly steeper than the MA/SL gradient means that the translocation gradient itself must also be significantly steeper than the MA/SL gradient. Unfortunately, by the nature of the method of obtaining the translocation gradient, it is impossible to compute fiducial limits for it directly.

Since, in the absence of a Maxwellian demon, transfer across cell membranes must be by a process of diffusion, the process of translocation must involve the setting up of different concentration gradients across the cell membrane at the

'entry' and 'exit' poles of the cell mediating the translocation, and the only type of process available for such a purpose is a reversible transformation within the cell of the substance translocated. This is the basis of Frank & Meyer's (1947) proposed 'osmotic diffusion pump'. Thus we have the situation that both translocation and utilization of glucose are manifestations of glucose metabolism, and we require to explain how the gradients of activity with respect to two aspects of the metabolism of the same substance can be entirely different.

We have no solution to this problem. But it is perhaps significant that the energy expenditure needed for translocation of glucose can be shown to be not more than 1 % of the glucose utilization, so that the observed relations would be consonant with the supposition that a specialized type of cell, forming a very small fraction of the total mucosal population, of the order of 1 %, was responsible for translocation. The translocation gradient would then be accounted for on the assumption of a graded population of such cells. As there is no histological evidence for such cells, there is no point in further elaboration of this hypothesis.

However, as we shall show in a later paper, there are gradients in the intestine of a translocative type which conform more or less closely to the MA/SL gradient, and the co-existence of such gradients with the much steeper glucose translocation gradient is most easily accounted for on some such hypothesis as that stated above.

Another finding of importance concerns the rate of absorption. In the experiments reported here it is known that the whole of each segment studied is exposed for a specified time to a specified concentration of glucose, so that a comparison of rates of absorption from the lumen in these experiments with rates of absorption in intact animals may be of value in indicating the difficulties of whole animal experiments on absorption rates. Westenbrink (1936), who has produced the most extensive series of estimates of glucose absorption in the intact rat in standard conditions, gives a mean rate of 440 ± 90 mg./hr. per rat of 141–205 g. body weight, following administration of 2 ml. 50 % glucose. There is absence of correlation between body weight and intestinal length (Fisher & Parsons, unpublished observations) so that it may be taken that the length of the small intestine in rats of the weights used by Westenbrink will range closely around the 110 cm. usually found in adult rats. If it were the case that the whole small intestine participated in the glucose absorption measured by Westenbrink, one would therefore estimate the mean rate of absorption as 4 mg./cm. intestine/hr. Since we have shown that when the lumen of the whole small intestine, except the duodenum, is exposed to 2·5 % glucose—one-twentieth of the concentration used by Westenbrink—the mean rate of absorption from the lumen is in the region of 4 mg./cm./hr., it is clear that gross dilution of the administered glucose, or confinement of absorption to a small region in the intestine, or both, must be supposed to occur in such experiments

as Westenbrink's. These conclusions are in agreement with those of Fenton (1945), who has demonstrated directly, *inter alia*, that gastric emptying time and passage of secretions into the intestine play important parts in determining the mean glucose concentrations in the intestine.

The demonstration that an appreciable fraction of the glucose 'absorbed' from the intestinal lumen is utilized in the intestinal wall also has a bearing on the interpretation of whole animal experiments. Since the proportion of the 'absorbed' glucose which is utilized in the intestinal wall is much higher at the ileal than at the jejunal end of the intestine, the physiological interpretation of an observed rate of 'absorption' clearly depends on a knowledge of the location of the absorption.

Finally, the high rates of glucose utilization in the intestine suggest that this process may be a significant contributor to the specific dynamic action of glucose.

SUMMARY

1. The surviving rat small intestine preparation translocates glucose from the lumenal to the serosal side of the mucosa against a concentration gradient, and this activity is inhibited by phlorrhizin.

2. There is a linear gradient along the intestine of ability to absorb glucose from the lumen, which can be specified quantitatively and can be separated into two components, a linear gradient of utilization of glucose by the tissues of the intestinal wall and a linear gradient of translocation of glucose across the mucosa.

3. It is suggested that the utilization gradient can be accounted for in terms of the gradient along the intestine of mucosal area per unit length of intestine, i.e. that glucose utilization per unit mucosal area is constant. The translocation gradient is much steeper than the utilization gradient, and cannot be accounted for in similar terms.

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THE EFFECT OF SOME SYMPATHOMIMETIC AMINES ON THE ASCORBIC ACID CONTENT OF RATS' ADRENAL GLANDS

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The cortex of the adrenal gland was shown by Szent-Györgyi (1928) to contain a relatively large store of ascorbic acid. The significance of its presence has remained uncertain, but recent work has suggested that it is associated with the secretion of cortical hormones.

In 1931, Hartman, Brownell & Crosby showed that the survival times of adrenalectomized rats were considerably reduced, if they were exposed to low temperatures. Moreover, injections of cortical extract were demonstrated to afford some protection against such exposure. It has since been shown that the ascorbic acid content of the adrenal is considerably reduced under the influence of low temperature (Sayers & Sayers, 1945), heat (Sayers & Sayers, 1947), haemorrhage (Sayers, Sayers, Liang & Long, 1945), and scalding (Ludwig & Chanutin, 1947). These conditions and many other injurious influences ('stresses') have been shown also to produce a depletion of the adrenal content of cholesterol (Levin 1945; Sayers *et al.*, 1945; Ludwig & Chanutin, 1947; Vogt, 1947).

In 1945, Long & Fry investigated the effect of subcutaneous injections and intravenous infusions of adrenaline on the ascorbic acid and cholesterol levels in the adrenal gland. Both levels were shown to fall, under the influence of this drug, but it was claimed that no depletion of either ascorbic acid or cholesterol occurred in hypophysectomized animals treated with adrenaline. In 1944, Vogt had shown that adrenaline considerably increased the amount of cortical hormone in the adrenal effluent and, later, in 1945, that the drug caused hypertrophy of the gland.

Sayers *et al.* (1946) showed that the depletion of the adrenal content of ascorbic acid and cholesterol, seen when an animal is subjected to stress, could

also be produced by injections of the adrenocortico-trophic hormone of the anterior pituitary. In conjunction with this, the evidence of the previous authors suggests that the depletion of the adrenal content of these substances is indicative of an output of cortical hormones.

The present work investigates more thoroughly the effect of adrenaline and other sympathomimetic amines on the adrenal content of ascorbic acid in an attempt to determine its significance.

METHODS

Administration of drugs. Groups of eight or more male rats of the Wistar strain were used, and were maintained on the full stock diet used in Dr K. H. Coward's colony. Injected and control animals were taken from the same litter. Each test animal received a single subcutaneous injection of the drug in its flank, while the controls received an injection of normal saline of equal volume. The animals were killed at times varying from 20 min. to 6 hr. after the injections, while the controls were taken at times between those chosen for the animals under test.

Extraction of glands. The animals were killed by a sharp blow on the back of the head. The abdomen was opened immediately and both glands removed as quickly as possible. All superfluous tissue was carefully removed before the glands were weighed. This process occupied approximately the same time in each case, so that any loss of weight due to drying would be reasonably constant. Weighing was accurate to 0·0001 g., the last figure being carefully determined, as a small error in the weight produced a gross error in the result.

After weighing, each gland was dropped into a test tube containing 10 ml. of 6% trichloracetic acid and a little acid-washed sand. With the aid of a glass rod, having an end shaped to fit the bottom of the tubes, each gland was ground up with the sand. The disintegration obtained by this method was sufficient to allow complete extraction of the ascorbic acid. The ascorbic acid content of each extract was then estimated precisely as described by Roe & Kuether (1943). For purposes of comparison each value was calculated in terms of mg. ascorbic acid/100 g. gland.

Comparison of effects of L-*adrenaline,* DL-*noradrenaline and* p-*sympatol on the rat's blood pressure.* Large rats of the Wistar strain, weighing 245 g. or more were anaesthetized with urethane and the carotid artery and jugular vein cannulated. Heparin was given (1 mg./100 g. body weight), and the blood pressure recorded from the carotid artery. An estimate was made of the ratios between the equipressor doses of *p*-sympatol, L-adrenaline and DL-noradrenaline.

RESULTS

The effect of sympathomimetic amines on the ascorbic acid content of the adrenal glands in normal rats

L-*Adrenaline*

The L-adrenaline was administered subcutaneously in doses of 0·005, 0·010, 0·020 and 0·040 mg./100 g. body weight; the quantities of ascorbic acid in the adrenal glands were estimated at times after injection varying from 1 to 4 hr.

At all dose levels, the maximal decline in the amount of ascorbic acid in the glands was found between 1 and 2 hr. after injection. Four hours after dosage, the return to the resting concentration was half-way towards completion.

Up to a dose level of 0·020 mg./100 g. body weight, there was a gradually increasing depletion of the vitamin, a dose of 0·005 mg. depleting it to 86% of the resting content, 0·010 mg. to 72% and 0·020 mg. to 47%. Doubling the

dose again (i.e. 0·040 mg./100 g.) depleted the ascorbic acid to 45% of the resting amount. This represented only an insignificant difference from the 47% obtained with 0·02 mg./100 g.

Details of the results are given in Table 1, and they are depicted graphically in Fig. 1.

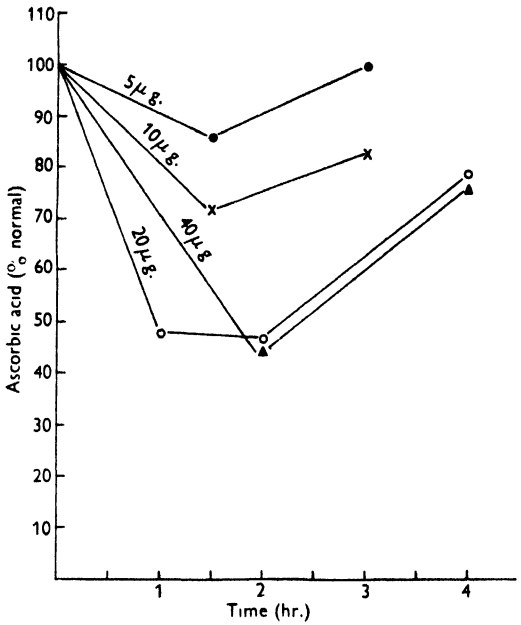

Fig. 1. The effect of various doses of L-adrenaline on the ascorbic acid content of the adrenal cortex of normal rats.

DL-*Noradrenaline*

The effect of doses of 0·015, 0·045, 0·060 and 0·090 mg. of DL-noradrenaline/100 g. body weight was investigated at time intervals varying from 1 to 3 hr. Doses of 0·015 and 0·045 mg./100 g. body weight did not cause a fall in the ascorbic acid content of the adrenal. Doses of 0·060 and 0·090 mg. produced a fall to 72% of the normal value 1 hr. after administration. This suggests that the fall occasioned by a dose of 0·060 mg. is maximal, but the absence of a figure for the ascorbic acid content of the gland 2 hr. after a dose of 0·090 mg. renders it inconclusive.

The time-relationship was the same as with L-adrenaline, the maximal effect being seen between 1 and 2 hr. after injection. The return to the resting level was quicker with the 0·060 mg. dose than with the 0·090 mg. dose, and was almost complete 3 hr. after administration of the former. This tendency of the small doses to produce a more transient effect was also noted in experiments with L-adrenaline.

The results obtained are given in detail in Table 1.

p-*Sympatol*

As the available quantities of this drug were limited, it was tested only at two dose levels. Doses of 12 mg./100 g. body weight caused a fall in the adrenal ascorbic acid to 53 % of the resting value, 1 hr. after the injection of the drug. Three hours after injection, the level had returned to 73 % of the resting value. A dose of 2 mg./100 g. body weight produced only a small reduction of the ascorbic acid content of the adrenals. The suggestion is that this is very nearly the threshold dose for *p*-sympatol.

Detailed results appear in Table 1.

TABLE 1. The effects of various doses of a number of sympathomimetic amines on the ascorbic acid content of the adrenal glands of normal rats

Drug	No. of estimations	Dose (mg./100 g. body wt.)	Time interval (injection to sacrifice) (hr.)	Vitamin C (mg./100 g. gland)	Vitamin C (percentage of normal)
Control	77	—	—	336±55	100
Adrenaline	2	0·005	1½	289	86
	2	0·005	3	337	100
	4	0·01	1½	241±20·3	72
	4	0·01	3	278±73·6	83
	4	0·02	1	161±6·0	48
	4	0·02	2	158±4·4	47
	4	0·02	4	266±27·4	79
	2	0·04	2	150	45
	2	0·04	4	258	76
Noradrenaline	2	0·015	1	309	92
	2	0·015	2	327	97
	4	0·045	1	324±18	96
	2	0·045	2	321	95·5
	2	0·045	3	322	95·5
	2	0·06	1	243	72·0
	2	0·06	2	224	67
	2	0·06	3	324	96
	4	0·09	1	241±29·0	72
	4	0·09	3	292±33·3	89
p-Sympatol	4	2·0	1	300±57·8	89
	4	2·0	2	316±38·8	96
	5	12·0	1	177±14·8	53
	6	12·0	3	262+41·2	78
Amphetamine	4	1·26	1	182+32·5	54
	2	1·26	2	164	48
	4	1·26	3	164±52·7	48
	2	1·26	6	331	98·5
	2	2·0	1/3	273	81·0
	6	2·0	1	223±31·4	66·0
	2	2·0	2	197	59·0
	4	2·0	3	214±76·3	63·0
	2	2·0	6	272	81·0

Amphetamine

Two dose levels were employed to investigate the effect of amphetamine; one at 2 mg./100 g. body weight, the other at 1·26 mg. The effects of both doses were noted at timed intervals after injection varying from 20 min. to 6 hr. With the 2 mg. dose the maximal effect was seen 2 hr. after injection of the

drug, while the return to the resting level was more delayed than had been the case with the other sympathomimetic amines. The 1·26 mg. dose produced a fall in the adrenal content of ascorbic acid which reached a maximum 2 hr.

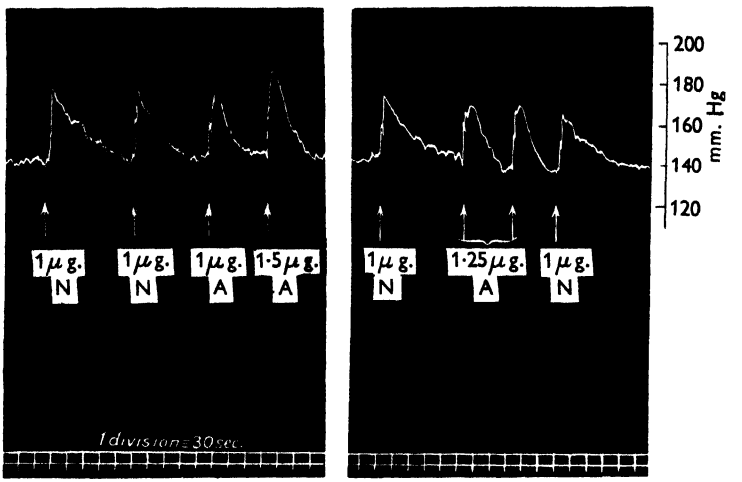

Fig. 2. Comparison of the effects of L-adrenaline and DL-noradrenaline on the rat's blood pressure. A = adrenaline; N = noradrenaline.

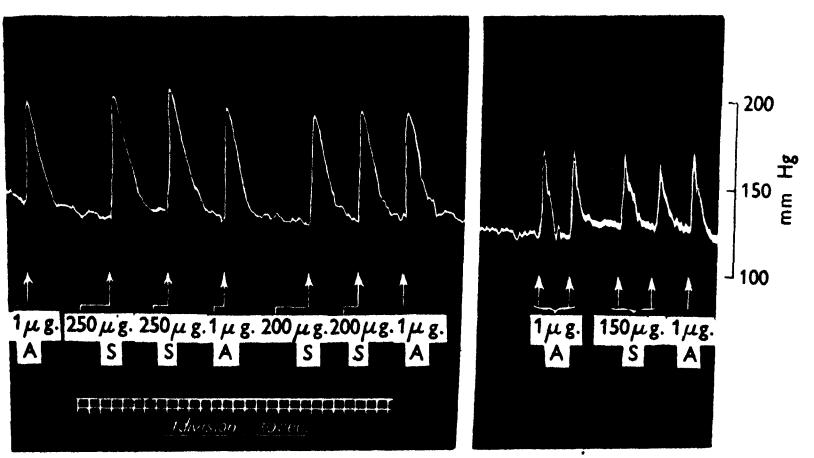

Fig. 3. Comparison of the effects of L-adrenaline and *p*-sympatol on the rat's blood pressure. A = adrenaline; S = sympatol.

after injection. The return to the normal value was more rapid than with the 2 mg. dose. The central effect of the drug was evident at both these dose levels, and manifested itself in rapid and aimless movements of the forepaws.

The results obtained in these experiments are summarized in Table 1.

Comparison of the effects of L-*adrenaline,* DL-*noradrenaline, and of* p-*sympatol on the rat's blood pressure*

DL-Noradrenaline and L-adrenaline were compared first. A dose of 0·001 mg. of DL-noradrenaline was shown to give a greater rise of blood pressure than 0·001 mg. of L-adrenaline, to be less active than 0·0015 mg. and equal to 0·00125 mg. of the latter. However, only the laevo isomer of noradrenaline produces a rise of blood pressure. The effective dose, in this respect, is, therefore, only half that of the racemic mixture employed, so that the ratio of dose of L-noradrenaline to the equipressor dose of L-adrenaline in the rat is 0·4.

The effect of *p*-sympatol was investigated in a similar way. A dose of 0·0250 mg. of *p*-sympatol was shown to produce a greater rise of blood pressure than 0·001 mg. of L-adrenaline, 0·200 mg. was shown to have the same effect, and 0·150 mg. to have a smaller effect than 0·001 mg. of the latter. Thus the ratio of equipressor doses of *p*-sympatol and of L-adrenaline is 200:1.

Figs. 2 and 3 illustrate these results.

DISCUSSION

In the foregoing experiments, all the sympathomimetic amines used caused a decline in the adrenal content of ascorbic acid; this was maximal between 1½ and 2 hr. after their administration. As far as these experiments go, increase of the dose of adrenaline beyond 20 μg./100 g. did not prolong or intensify the depletion in the adrenal ascorbic acid. Such limitation may be due to an equilibrium reached between disappearance and replacement of the ascorbic acid from the blood stream. If the ascorbic acid plays a part in the chemical processes leading to synthesis of the cortical hormones, it is also possible that its consumption is limited by some other metabolite essential for the synthesis.

The sympathomimetic amines used may be divided into two groups with respect to their pressor effects and their reduction of the adrenal ascorbic acid content: those which produce both effects in small doses, and those which only evoke them in much greater doses. It may be inferred, therefore, that the dose of a sympathomimetic amine required to deplete the adrenal ascorbic acid will be of the same order as that required to produce a pressor response. Assuming that only L-noradrenaline is effective in producing a fall in the adrenal content of ascorbic acid, then the doses of L-adrenaline, L-noradrenaline and *p*-sympatol causing similar falls in ascorbic acid are in the ratio of 1:45:1200, whereas their equipressor doses are in the ratio of 1:0·4:200.

SUMMARY

1. Though L-adrenaline, DL-noradrenaline, *p*-sympatol and amphetamine each produced a fall in the ascorbic acid content of the adrenals of normal rats, L-adrenaline was the most effective. The relationship between equipressor doses

of L-adrenaline, L-noradrenaline and *p*-sympatol was 1:0·4:200; whereas, doses causing equal falls in the ascorbic acid content of the adrenal glands were in the ratio of 1:45:1200.

2. Gradation of dose of L-adrenaline gave gradation of effect up to a maximal figure.

3. The maximal fall in the adrenal content of ascorbic acid occurred between 1 and 2 hr. after the administration of the drugs.

I wish to thank Prof. G. A. H. Buttle and Dr M. Vogt, who gave me much kindly advice and criticism. The work was done while holding the Allen and Hanbury Research Fellowship. It was presented as part of a thesis for the degree of Doctor of Philosophy in the University of London.

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CATION CONTROL IN HUMAN ERYTHROCYTES

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It is well known that during cold-storage human erythrocytes lose K and gain Na in accordance with their respective concentration gradients. References to this work are given by Maizels (1943), who also shows that total cell base increases. If, now, these cold-stored cells are incubated cations move against the concentration gradients, their passage being activated by glycolysis, so that K enters the red cell and Na leaves (Harris, 1941). This work was extended by Maizels (1948; 1949) who has shown that loss of Na exceeds gain of K so that cell base and volume which have risen during cold-storage tend to return toward the normal during incubation and may even fall below it. Output of Na was shown to be active, and uptake of K either active or else passive and secondary to output of Na. It was further found that active cation movements in unbuffered solutions were maximal when the pH at the end of incubation was between 7·4 and 7, but owing to acid formation during incubation the former corresponds to an initial pH of about 8·2 so that the optimal pH may lie anywhere between 8·2 and 7. The present paper seeks to define these matters more clearly and also to define the effects of plasma Na and K concentrations on the active output of Na. It is therefore divided into the following sections: (*a*) The demonstration that uptake of K during incubation is not active but passive. (*b*) The investigation of the effects of external Na concentration on the active output of Na by erythrocytes. (*c*) Examination of the influence of external K on Na output. (*d*) The definition of the pH range of active cation movements in cells mixed with buffer solutions. In addition, it has seemed desirable to attempt to distinguish between real and apparent cation movements: thus, in the case of Na there is a real active output from the erythrocyte which is opposed by passive diffusion into the cells in accordance with physical factors; in so far as the former exceeds the latter so does output become apparent.

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Therefore, if the real output of Na is to be assessed, it is necessary to have a measure of passive diffusion, and this added to the apparent value would give the real value. An attempt to accomplish this is described below.

The principle of the method used is as follows: blood is cold-stored with solutions containing varying amounts of NaCl, tonicity being kept constant by the addition of LiCl. Since during cold-storage passive diffusion alone occurs, cell Na will vary with the external concentration and may be made at will either high or low. At the end of cold-storage these bloods are appropriately modified by further additions so that after incubation the effects of variations in external Na and K or of pH may be observed. All bloods are finally centrifuged: packed cells from cold-stored blood give the cold-storage levels; those from bloods cold-stored and then incubated with glucose, the active incubation levels; cells from blood incubated without glucose give the passive incubation levels. All these are compared with the findings in packed cells from the original fresh untreated blood which is used as the standard for changes in cell volume and cation contents.

METHODS

These are modifications of those used elsewhere (Maizels, 1949).

Mechanism of potassium uptake. Heparinized blood was divided into four 20 ml. lots, two lots being mixed with 40 ml. NaCl solution (0·15 N) and two with 40 ml. LiCl solution (0·15 N); to one of each pair glucose was added to 2%, the corresponding sample being glucose free. After 6 days' cold-storage half of each sample was centrifuged to provide the cold-storage base-line values, while the several residues were incubated with 2 ml. Na_2HPO_4 $2H_2O$ and 5 ml. KCl (0·15 N); further, to the glucose-containing samples 0·38 ml. NaOH (0·45 N) were added and to the glucose-free samples 0·38 ml. NaCl (0·45 N), while to all samples 2 ml. water were added to restore the total cation content of the incubation plasma to the cold-storage level. After 20 hr. incubation all bloods were centrifuged and the cell deposits analysed. Lithium was taken as the difference between total base (determined electrolytically) and the sum of Na + K; it has an absolute error of ± 4 m.equiv./l. In this way two systems have been examined, one with high cell and plasma Na permitting free active and passive movements of Na and one with low Na values and restricted movements. The corresponding movements of K have been examined under these conditions.

Relation of cell and plasma sodium to active sodium output. The object here is to prepare sets of cells with varying Na concentrations, to incubate these in plasmas of varying Na concentrations and to see the effects of variations in cell and plasma Na on Na output. Citrated blood was mixed with 2 volumes of the following solutions: NaCl (0·15 N), equal parts of NaCl and LiCl (0·15 N) and LiCl (0·15 N). Glucose was added to 2%. After 6–8 days' cold-storage the Na concentrations of the corresponding cells were respectively high, medium and low. Supernatant plasmas were now removed and the cells from each sample distributed as follows: one part was used as the cold-storage control and the rest divided and incubated for 24 hr. with 8–12 volumes of diluents whose Na contents varied but whose tonicity was maintained by the addition of appropriate amounts of LiCl. All diluents contained KCl, NaOH and phosphate as in the previous section and before the addition of cells sterile glucose was added to 0·5%. It should be noted that as a result of the technique used, cells with high Na values at the end of cold-storage have plasmas that are relatively rich in Na at the end of incubation. This arises as follows: the Na content of cold-stored cells is varied by varying that of the corresponding plasma; when the settled cells are transferred to the incubation fluid, there is a 'carry over' of 1–1·5 ml. intercellular plasma which will enrich or impoverish the incubation fluid. Further, during incubation Na-rich cells discharge much more Na into the external solution than do Na-poor cells. Hence, when cells are mixed with large volumes of

incubation solutions all of the same Na content, these solutions at the end of incubation show slight differences in their Na contents which vary directly with the contents of the added cold-stored cells.

Effects of potassium on the active movements of cations. 10 ml. lots of cold-stored citrated blood were incubated for 20 hr. with phosphate, NaOH and 20–25 ml. of a solution containing varying amounts of Na and K but with a total normality of 0·15; in some cases parallel experiments were conducted without as well as with glucose.

pH *and active cation movements.* Plasma was dialysed to remove glucose and the salt content restored by adding NaCl. Next, $Na_2HPO_4.2H_2O$ (0·1 M) was added in the proportion of 1 to 6·5 and the solution 'cleared' through paper pulp. To the heparinized filtrate KCl was added in the proportion of 1 to 7 and the product Seitz-filtered into large sterile bottles. Acid or alkali was added to give the appropriate pH. Experiments were done in parallel: to one batch 10 ml. cold-stored citrated blood (1 part of 3% trisodium citrate to 9 of blood) containing glucose was added together with a further 2·5 ml. glucose (50%); to the other batch of bottles glucose-free blood was added. Bottles were then incubated flat for 6 hr. after which the film of settled cells was collected, centrifuged and the packed cells analysed.

Three general points common to all experiments require further comment:

(1) Na and K contents are corrected for changes in cell volume by reference to unit volume of fresh unstored and unincubated cells.

(2) Concentrations of cell and plasma cation. Previously it was assumed (Maizels, 1949) that an average value for cell water sufficed to calculate cation concentrations. The composition of normal plasma varies but little, the cation content of cells from fresh blood varies between 105 and 125 m.equiv./l. with an average of 115. Since plasma and cells are in equilibrium, it follows that the cation concentration (though not the content) of fresh untreated erythrocytes must also be fairly constant and hence that cell water varies directly with the cation content; both, in fact, are determined by the haemoglobin content of the cells (Maizels, 1936). Cell water, then, has been calculated on the basis that water in fresh cells averages 69·5% wt./vol. and cation, 115 m.equiv./l. Hence, if a batch of fresh cells has a cation content of 110 m.equiv., water is taken as 69·5 × 110/115 or 66·5%; should the volume of these cells increase by 15% during storage the water content will now be (66·5 + 15)/115 or 70·5%. Calculation of plasma cation is carried out as described elsewhere (Maizels, 1949). Errors in the method for cell-Na and K concentrations are ±3% and for plasma concentrations ±6%. It follows that the concentrations of cell and plasma base are mainly of use in affording a clear but approximate indication of the concentration gradients with or against which cations move.

(3) Apparent and real cation movements. Estimation of these involves the measurement of the amount of Na which would enter the erythrocyte and of K which would leave in the absence of active movements, and this in turn involves the inhibition of glycolysis. Fluoride and iodoacetate were found unsuited to the purpose for their inhibitory effect increases progressively with the dose used, as did the haemolysis which was the usual accompaniment. Inhibition of active movements was finally attained by omitting glucose from a control sample both during cold-storage and incubation; this gives the passive incubation level. The difference between the cold-storage and active incubation levels gives the apparent output of Na, while that between the passive and active incubation levels might be expected to equal the true turnover. Unfortunately, the passive incubation samples are not perfect controls because: (*a*) cold-storage in the absence of glucose may adversely affect the nutrition and hence the permeability of erythrocytes. This effect is probably slight since, although there occurs during cold-storage a leakage of Na into the cells and of K out, this leakage is practically the same in cells stored with and without added glucose—at least for 6 or 8 days. (*b*) Interference with cell nutrition in glucose-free bloods, though slight during cold-storage, may well be more marked when glucose is absent during incubation: thus haemolysis in incubated glucose-free systems is perceptibly more than in glucose-containing systems (where it is often absent), and this may well have an adverse effect on permeability. (*c*) Bloods cold-stored without added glucose might still contain a residue of their original natural glucose, and this might permit a small degree of active cation movement during incubation. In practice, such residual glucose is insignificant (Maizels, 1949). (*d*) Bloods incubated with glucose become much more acid during

incubation, and this acidity inhibits active movements. Alkali is therefore added to the glucose-containing systems just before incubation until the pH is about 8. On incubation this shifts to about 7. Glucose-free systems without added alkali usually have a pH of about 7·1 at the end of storage and of 7 after incubation. Thus, although both systems reach roughly the same pH at the end of incubation, in the absence of glucose the shift is small, while in the presence of glucose it is considerable, and so the pH conditions of the two systems are not truly comparable. (*e*) The concentration gradient: the effects of this are discussed later.

RESULTS

Mechanism of sodium output and potassium uptake

To investigate this matter, one blood was stored and incubated with Na-rich plasma and a second with plasma poor in Na but rich in Li. At the end of cold-storage, Na in the cells of the first blood was high and in the second, low. Clearly, if there is uptake of K by the cells of the first blood where output of Na is large and active and not by the cells of the second blood where by virtue of the low cold-storage level, significant output of Na during incubation is not possible, then uptake of K, when it occurs, must be passive and secondary to output of Na. Experimental details are given in the section on methods and results are shown in Table 1.

TABLE 1. Incubation and exchange of sodium and potassium in sodium-rich and sodium-poor bloods

(Fresh original cells, K content 102 and Na 15 m.equiv./l.)

	Plasma			Cells								
	Concentration at start of incubation (m.equiv./l.)			Volume (% original)	pH at 20°	Contents (m.equiv./l.)			Concentration (m.equiv./l. cell water)		
Exp.	K	Na	Li			K	Na	Li	K	Na	Li
1*a* CSL G 0	6·5	139	0	104	7·02	64	56	0	86	75	0
b CSL G +	6·5	139	0	106	6·98	65	56	0	86	74	0
c PIL G 0	26	123	0	118	6·94	58	77	0	66	87	0
d AIL G +	26	123	0	100	6·86	88	20	0	126	28	0
e AIL G +	26	64	59	102	6·82	76	18	21	105	25	29
f CSL G 0	4·1	33	110	105	6·96	78	12	30	104	16	40
g CSL G +	4·1	33	110	108	6·95	81	9·7	34	103	12	43
h PIL G 0	25	42	83	118	7·03	76	18	43	86	20	49
i AIL G +	25	42	83	108	6·90	80	6	40	102	8	51

CSL = cold-storage level; PIL = passive incubation level; AIL = active incubation level. G 0 = no glucose added; G + = glucose added. Contents are referred to original cell volume.

If the data for Na-rich systems are examined it will be seen that during cold-storage Na rises and K falls with the concentration gradients and that total base (Na + K) and volume rise above the values in the fresh cells (1 *a*, *b*, Table 1). On incubation, erythrocytes from the sample containing no glucose (1 *c*) show an increased gain of Na, a small loss of K and a further rise in Na + K and in volume. The Na-rich systems to which glucose has been added, on the other hand, show a marked decrease of Na, a relatively smaller gain of K, in each case against the gradients and total base falls, as does cell volume (1 *d*).

In the lithium-rich sodium-poor bloods on cold-storage, Li increases in the erythrocytes and K falls, conforming in each case with the respective concentration gradients while Na falls *against* the gradient, the net result being a small increase in total base. Fall in cell K is less than in Na-rich bloods, presumably because the larger hydrated Li ion penetrates less freely than Na^+ and the escape of K in accordance with the concentration gradient is correspondingly restricted because of the osmotic requirements of non-penetrating cell anions (haemoglobin and organic phosphate).

At the end of cold-storage in Na-poor bloods then, cell Na is low, but even so there is a relatively great though absolutely small output of Na during subsequent incubation with glucose (cf. 1 *f*, *i*), the cell distinguishing between Li and Na so that the former so far from being expelled continues to increase with the concentration gradient. Under these conditions, that rise of cell K against the concentration gradient, which is observed in Na-rich systems, does not occur (cf. 1 *f*, *i*). Further, loss of Na from the cells of Na-rich systems incubated with glucose leads to a fall in cell total base and volume (1 *b*, *d*), whereas incubation of Li-rich systems leads to no such fall whether glucose be present or not (1 *f*–*i*).

Again, in another experiment cells were cold-stored in Na-rich plasma and then incubated in a Na-poor medium. Here, although cell Na at the start of incubation was high and active output marked, maintenance of cell base was mainly effected by the substitution for Na of Li (which entered the cell with the gradient) and much less by external K entering against the gradient (1 *e*); indeed, in some cases replacement of cell Na was effected entirely by Li. This also indicates that entry of K into red cells against the concentration gradient is secondary to active output of Na.

Another point emerges: on cold-storage of cells in plasma of high Na concentration (139 m.equiv./l.) the content of Na rises with the gradient (cf. Na in fresh cells and 1 *a*, *b*), while when external Na is only 33 m.equiv. Na leaves the cells even at low temperatures, against the concentration gradient, provided that the latter be not too steep. Further, it will be noted that during the cold-storage of glucose-free systems poor in Na, a small decrease of Na still occurs (cf. original cells and 1 *f*, *g*), and this must be ascribed to activation of ionic movements by a persistent remnant of the natural glucose present in fresh blood.

Relation of cell and plasma sodium to active sodium output

In most of our experiments we have been concerned with cells alone; in the present section our concern is with the precise relations of cells to plasma. Cells were suspended during incubation by a mechanical shaker in an incubating chamber and three samples of blood were so prepared as to give after cold-storage high, medium and low cell-Na contents. These were respectively incubated in media whose Na contents were high or low and sometimes high, moderate and low (see 'methods'). Results are shown in Table 2 and Fig. 1.

TABLE 2. Relation of cell and plasma sodium to sodium output

Exp.	Incubated plasma [Na]	Cold-stored cells: Na content (m.equiv./l.)	Cold-stored cells: [Na]	Incubated cells: Na content (m.equiv./l.)	Incubated cells: [Na]	Incubated cells: Cell [Na] / Plasma [Na]
1*a*	126	61	81	30	41	0·325
b	122	40	53	26	37	0·304
c	116	19	25	24·5	33	0·286
d	63	61	81	14·5	20	0·317
e	58	40	53	13	17·5	0·303
f	55	19	25	11·5	15·5	0·283
2*a*	127	65	84	24	35	0·277
b	121	44	56	21·5	30	0·248
c	118	33	42	18	27	0·228
d	63	65	84	12·5	17·5	0·278
e	59	44	56	10	14	0 238
f	55	33	42	8·5	12	0·219
3*a*	124	41	53	17	23	0·186
b	120	22	30	15·5	20	0·166
c	116	10	13	13	17·5	0·152
d	63	41	53	8·5	12·5	0·197
e	59	22	30	7	10	0·170
f	54	10	13	6·5	8·5	0·160
4*a*	130	58	74	15	20·5	0·157
b	128	27	34	12	16	0·125
c	125	13	16·5	11·5	15	0·120
d	96	58	74	12	16	0·167
e	93	27	34	8·5	11·5	0 125
f	90	13	16·5	8·0	10·5	0·117
g	60	58	74	8·0	10·5	0·174
h	58	27	34	6	8	0·136
i	54	13	16·5	5·5	7	0·128
5*a*	134	65	77	20·5	26·5	0·198
b	130	35	43	17	23	0·177
c	127	14	17	16	20	0·157
d	97	65	77	16	20 5	0·212
e	94	35	43	12	15·5	0·164
f	91	14	17	10·5	13·5	0·148
g	62	65	77	10	12	0·194
h	58	35	43	7·5	9	0·157
i	55	14	17	7	8	0·146

Changes in cell K have been discussed in the preceding section. Na changes were as follows: when cells of the *same* cold-storage contents were incubated in solutions of *varying* Na concentrations, Na output varied inversely with the external concentration and the level to which cell Na fell after incubation varied directly with the plasma level (compare records *a* with *d* and *g*; *b* with *e* and *h*, etc., Table 2). This is to be expected, for the higher the external Na, the greater will be the passive backflow and the less the apparent output. When cells of *varying* cold-storage Na contents were incubated in solutions of the *same* Na concentrations (records *a–c*; *d–f*; *g–i*) it was seen that within each experiment the higher the cold-storage value, the greater was the output of Na. This also is to be expected for the higher the initial cell Na, the less is the

contrary backflow with which active output has to contend. As a result of the very high output of Na when cells of high sodium contents are incubated and the small output when initial cell Na is low, cells of varying Na contents at the beginning of incubation tend to approach the same low values at the end. Thus in Exp. 2 (Table 2) cell Na concentration falls by 49 m.equiv. to 35 in record *a*, and by 15 m.equiv. to 27 in record *c*, and there is a suggestion in Table 2, that values for cell Na at the end of incubation are tending to approach a constant relation to the plasma concentration. This matter is discussed again later.

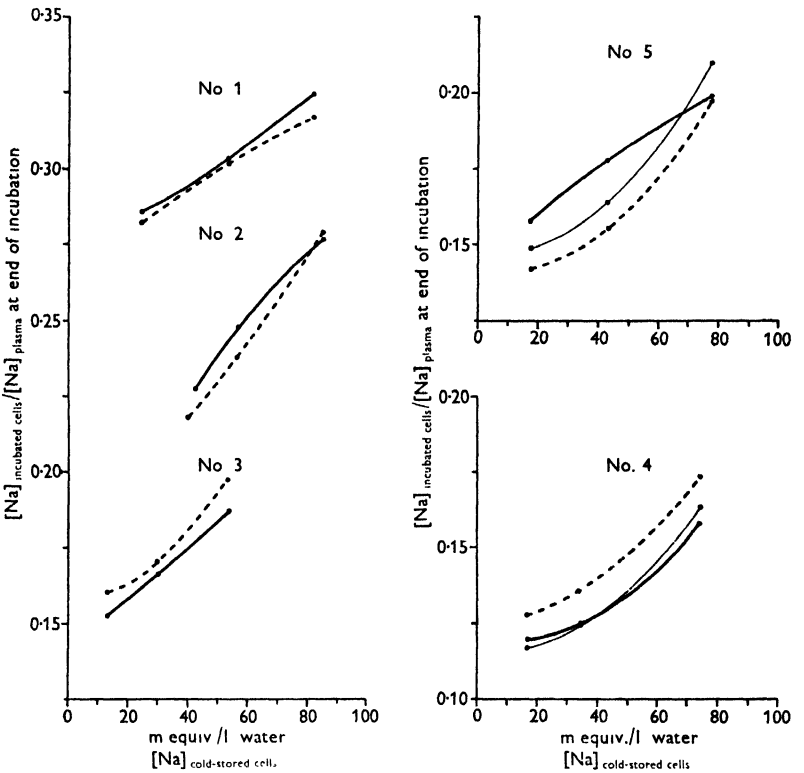

Fig. 1. Relation of $[Na]_{cells}/[Na]_{plasma}$ at end of incubation to $[Na]_{cells}$ at end of cold storage. ——, cells in plasma of high Na concentration; ——, cells in plasma of medium Na concentration; - - - -, cells in plasma of low Na concentration.

Effects of plasma potassium on the active movements of cations

The technique by which these were studied is described in the section on methods. In some cases cells were washed with saline before incubation to remove intercellular K. Results are shown in Table 3. The following observations may be made:

(1) During incubation in the presence of glucose K enters the erythrocytes and Na leaves in each case against the concentration gradient. Uptake of

K does not balance but is less than the output of Na. Thus, with a plasma K of 24 m.equiv./l., cell K rises by 14 m.equiv., from 71 to 85 m.equiv.; and 23 m.equiv. Na are discharged, while with an external K of 3·4 m.equiv. only

TABLE 3. External potassium and cation movements

(Cold-storage 6 days; incubated 20 hr. in Exps. 1 and 2; 6½ hr. in Exp. 3.)

Exp.	Final plasma concentrations (m.equiv./l.) K	Final plasma concentrations (m.equiv./l.) Na	Volume (% original)	pH at 20°	Cells: Contents (m.equiv./l.†) K	Cells: Contents (m.equiv./l.†) Na	Cells: Concentrations (m.equiv./l. cell water) K	Cells: Concentrations (m.equiv./l. cell water) Na
1*a* CSL	20	151	102	7·14	71	48	97	66
b PIL K +	26	145	111	7·20	69	63	84	77
*c** PIL K −	1·5	172	104	7·13	57	67	76	89
d AIL K +	24	148	98	7·12	85	25	124	36
e AIL K +	9·8	165	92	7·03	83	25	132	40
f AIL K +	5·3	172	93	7·02	79	30	124	47
g AIL K +	3·4	174	91	7·00	74	32	120	52
*h** AIL K −	0·5	174	95	7·13	67	48	102	73
2*a* CSL	25	141	105	7·22	82	43	106	56
*b** PIL K +	27	128	120	7·15	82	61	89	67
*c** PIL K −	0·8	155	114	7·05	72	66	84	77
*d** AIL K +	25	132	102	7·10	97	21	132	29
*e** AIL K +	5	152	99	7·01	92	26	130	36
*f** AIL K +	2	153	97	7·01	81	34	118	50
*g** AIL K −	0·6	156	97	7·07	75	43	108	62
3*a* CSL	22	150	104	7·18	72	51	99	70
b PIL K +	3·8	162	109	7·36	74	54	95	70
c PIL K +	1·8	164	110	7·38	72	54	91	68
d PIL K +	1·2	165	108	7·38	70	56	91	73
e PIL K −	1·0	165	109	7·37	68	57	87	76
f AIL K +	3·5	162	103	7·30	86	34	119	47
g AIL K +	1·6	164	99	7·28	82	36	120	53
h AIL K +	1·1	165	99	7·31	78	39	114	57
i AIL K −	0·8	165	99	7·29	73	43	107	63

CSL = cold-storage level. PIL = passive incubation level; no glucose added. AIL = active incubation level; glucose added. K + = K added before incubation. K − = no K added.

* = cells washed with saline before incubation.

† = contents referred to original cell volume.

In Exps. 1 and 3 phosphate was present in the plasma as a buffer. Plasma in Exp. 3 was dialysed and reconstituted.

3 m.equiv. K enter the cell and 16 m.equiv. Na leave (contents column, Table 3, records 1 *a*, *d*; 1 *a*, *g*). Further, when plasma K falls to a very low level apparent output of Na is much reduced and may cease altogether (1 *a*, *h*; 2 *a*, *g*; 3 *a*, *i*). It will be shown later that though apparent output of Na ceases, real output probably persists.

When active movements are suppressed by the omission of glucose, cell Na rises, but there is little loss of K during 20 hr. incubation (Table 3, 1 *a*, *b*; 2 *a*, *b*; 3 *a*, *b*) except in those systems designed to contain practically no external

K and even here loss of K is quite small—14 m.equiv./l., in Exp. 1 (*a*, *c*); 10 in no. 2 (*a*, *c*) and 4 in no. 3 (*a*, *e*).

(2) During incubation in the presence of glucose, cell volume and the content of base fall, although the external base concentration remains practically unaltered, at least in Exp. 1. Further, even when external K is so low that apparent output of Na falls, the cell content of Na + K is still below the cold-storage level (1 *a*, *g*; 2 *a*, *g*; 3 *a*, *i*).

In glucose-free systems cell base and volume rise during incubation, provided, as is usually the case, that non-penetrating anion within the cells exceeds that in the plasma (Table 3, Exps. 1 and 2). But in Exp. 3, where slowly penetrating inorganic phosphate was added to the external phase, rise in total base during incubation without glucose is slight. When increase of base does occur, it is most marked when external K is plentiful (see Table 3; passive incubation levels, records 1 *b*, *c*; 2 *b*, *c*; 3 *b*, *e*).

pH *and cation movements*

This investigation presents two difficulties. First, the apparent movement of Na or K is the resultant of a true active movement against the gradient and of passive movement with the gradient, and these may each be affected differently by pH; thus, the pH of maximum apparent movement is not necessarily the same as that of true active movement. In order to attempt the definition of both points it is necessary to conduct experiments with and without glucose at various pH. Curves obtained from glucose-containing systems show active movements and the differences between these and the cold-storage base-line values for Na and K give the apparent changes in cation distribution on incubation. Curves obtained with glucose-free bloods give the incubation values for passive diffusion. The second difficulty arises from the liberation of acid during incubation with glucose. Unbuffered systems may shift from pH 8 to 7, and it is not possible to say at which point active cation movements are maximal. Theoretically this might be achieved by means of a 'time curve', but in practice this is unsatisfactory because active movements persist over a wide range and the increments are too small and the experimental errors too great to permit of precise identification. Nor was the use of buffers wholely satisfactory. Thus, on incubating 1 volume of cells with 50 of plasma, the shift in pH was considerable after 24 hr. When plasma was replaced by phosphate (0·2 M) pH was more stable, the shift being 0·15 at pH 7·4 and 0·3 at pH 8. This method gave results in good agreement with the more physiological technique finally used of mixing 1 part of phosphate buffer with 6 parts of plasma and incubating 50 parts of this with 1 of cells for only 6 hr., at which time active movements were well marked. The pH shift under these conditions was quite small. Details of procedure are given in the section on methods.

The effects of incubating blood with plasma-phosphate buffer are shown in Fig. 2. The simple diffusion data are as follows:

(*a*) Acid shift in the absence of glucose is slight: less than 0·05 pH. The shift is probably due to the level of slowly penetrating inorganic phosphate in the plasma being greater at the beginning than at the end of incubation, and perhaps to the persistence of traces of glucose and of glycolysis in the so-called glucose-free systems.

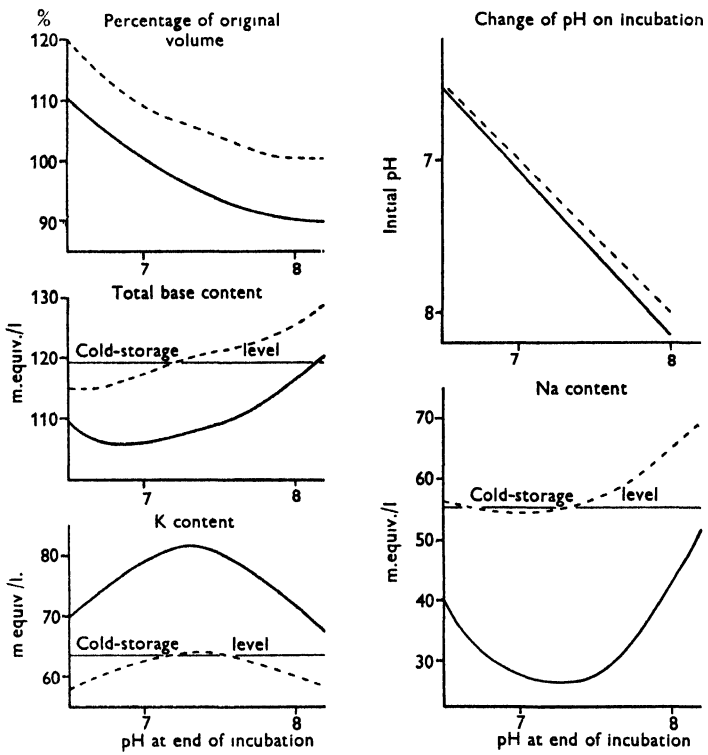

Fig. 2. Effects of incubation on pH, K uptake, Na output, total base and volume of erythrocytes. Cells in phosphate-plasma solution, 6 hr. Concentrations: K = 15, Na = 146 m.equiv./l. ———, glucose added (active incubation); - - - -, glucose not added (passive incubation).

(*b*) Little Na diffuses into the erythrocytes and little K escapes during the short period of incubation, except at high pH where Na rises and K falls, suggesting increased permeability to cation at high pH. Similar changes, probably due to cell damage, are seen below pH 6·5.

(*c*) Cell volume increases with fall of pH because less cell base is combined with haemoglobin and more with osmotically active anions.

(*d*) Total base, Na + K, increases with rise of pH owing to increase of base bound by cell Hb. Intra- and extracellular base concentrations are about the

same at pH 7·4 when cell Hb^- and phosphate$^-$ are presumably balanced by the slowly diffusing phosphate$^-$ of the plasma.

In glucose-containing systems the following observations may be made at 6 hr.:

(*a*) Acid shift is small particularly below pH 7·5.

(*b*) There is a considerable apparent output of Na over a wide range between 6·5 and 8·2 (difference between cold-storage and active incubation levels). Between pH 7 and 7·6 the base of the curve is almost flat with a probable minimum at about 7·3.

(*c*) There is a considerable uptake of K against the concentration gradient over a wide range with a maximum at about pH 7·3.

(*d*) The curve for the active incubation levels of total cation falls with pH to a flat base between 7·4 and 6·6. The cause for the general low level lies in active cation movements, with output of Na exceeding uptake of K. As a result of this, total base in samples incubated with glucose is less than in glucose-free systems or in the cold-stored controls. A further factor in the fall of total base at low pH is decreased base binding by haemoglobin. Increase of total base at very low pH (below 6·6) must be ascribed to failure of the Na output mechanism in this range. As a result minimum values for cell base are observed at about pH 7. Cell-base *concentration* rises progressively with pH.

(*e*) In accordance with the change in total base, cell volume on incubation with glucose is considerably less than when glucose is absent and at a corresponding pH 3–5% less than the volume of cold-stored cells.

It would, therefore, seem that active cation movements are most effective between pH 7·2 and 7·4 or, allowing for acid shift during incubation, between 7·2 and 7·45. They are, however, almost as effective over a much wider range—between 6·85 and 7·65.

DISCUSSION

It is now well known that the erythrocyte membrane is freely permeated by cations, yet during life the cation content of the cells is kept constant. This constancy is dynamic for there are some forces leading to passive inflow of cations into the red cells and others leading to active discharge. It is assumed that the sites of these movements in opposite directions are distinct; that there are areas for passive diffusion and others for active output. The concentration gradient tends to send Na into the cells and K out, while all ions are attracted by the non-penetrating anions within—haemoglobin and organic phosphate. Since, apart from these two, all other cations and anions are, with unimportant exceptions, permeant, the tendency to achieve an equilibrium of the kind described by Donnan will, unchecked, lead to a progressive entry of salts and water into the cells until ultimate rupture occurs. This process is opposed under physiological conditions by active output of Na, and also *in vitro* when blood is incubated with glucose.

If glucose be absent from the system, however, the ion-attracting properties of cell non-penetrating anion are clearly shown by a tendency for cell base and volume to increase during cold-storage and especially during incubation (see Table 1, 1 *a–c*; and Table 3, 1 *a*, *b*; 2 *a*, *b*). The tendency is slight in Exp. 3 (Table 3) because of opposition by the slowly penetrating inorganic phosphate added to the external phase. Attraction by cell non-penetrating anion is further shown by the slowness with which K escaped from cells even when cell K is high and plasma K low (Table 3, 1 *a–c*; 2 *a–c*; 3 *a–e*). Again, increase of cell base in glucose-free systems must always occur at the expense of plasma base. Thus, if batches of red cells are incubated with plasmas of decreasing K concentrations in the absence of glucose, fall of external K leads to increased loss of K from the cells, though rise in total base still occurs. If this rise were the same in all cases, increased passive entry of Na would be needed in low plasma K systems, but in fact though increase of cell base occurs in all cases, it is less in the low plasma K systems, presumably owing to the relatively slow penetration of Na^+. If, however, for any reason the cell is damaged, as by iodoacetate, permeability is much increased: fall in cell K is marked, but rise in cell Na is very marked and so cell base rises considerably (Table 4).

TABLE 4. Effects of iodoacetate on cation exchange

	Plasma Concentrations (m.equiv./l. water)			Cell				
							Contents (m.equiv./l.)	
	K	Na	Iodoacetate	Volume (% original)	pH at 20°	Lysis	K	Na
Cold-storage level	20	145	0	100	6·75	0	64	47
Active incubation level	26	132	0	97	6·64	0	79	24
Active incubation level	26	132	2·5	103	7·53	Trace	44	82
Active incubation level	26	132	5	111	7·40	Much	40	96

(Glucose added to all samples.)

That this increase of cell base so evident in glucose-free systems is opposed by active cation movements when systems are incubated in the presence of glucose is shown by the fall in total cell base which occurs (see especially, Table 1 and Table 3, Exp. 1, where plasma-base concentration was about the same during cold-storage and incubation). But since the fall in cell base is due to output of Na, this output must be active; indeed it could in no way be secondary to active uptake of K, for with such uptake against the gradient through active areas of the cell membrane, Na should still diffuse *into* the cell through passive areas of the cell membrane in response to the attraction of cell non-penetrating anion. Actually, Na is expelled even in the absence of K uptake (Table 3, 2 *a*, *f*; 3 *a*, *i*).

Uptake of K could still be either active or passive, and direct evidence in favour of the latter is given in Table 1, where cells are cold-stored with plasma poor in Na and rich in Li. Here the cells contain so little Na at the end of

incubation that output of Na, though relatively great, is absolutely small, only 3 or 4 m.equiv./l. (*g* and *i*); under these conditions there is no output of Li and no uptake of K. This shows that human erythrocytes distinguish between Na and Li, actively excreting only the former. In the absence of output of Na there is no call for reciprocal passive uptake of K, though if uptake of K were indeed active, some would still enter the cells. In fact, Li enters the erythrocytes and K leaves in conformity with their respective concentration gradients. It is of interest to note that active output of Na on incubation in the presence of glucose is much more rapid than passive input when glucose is absent. Thus in Table 3, Exp. 1, passive diffusion equals 15 m.equiv. in 20 hr., and active output is 23 (*a*, *b* and *d*) while in Exp. 3 the figures are 3 and 17 (*a*, *b* and *f*). It is this powerful outward drive of Na which causes the fall in cell base and volume during incubation with glucose and which is the *in vitro* expression of that mechanism which *in vivo* keeps cell volume and base constant. Cell base during *in vitro* experiments falls because Na output exceeds passive K uptake. It follows that electrical neutrality is maintained only in part by an exchange of cations and that some of the Na excreted from the cell must be accompanied to the plasma by diffusible cell anion.

It would thus appear that output of Na is dominant in maintaining the level of cell base, and the effects of other influences on this dominant factor were therefore studied—level of plasma and cell Na, plasma K and cell pH.

Relation of sodium output to cell sodium and plasma sodium

It may be assumed that in a system containing a large excess of plasma, if active output proceeded to equilibrium, the Na concentration, of the cells would be related purely to the plasma concentration, and there is indeed a suggestion in Table 2 (more especially, perhaps, in Exps. 1, 4 and 5) that under ideal conditions the value $[Na]_{cell}/[Na]_{plasma}$ might be constant. However, it is clear that under experimental conditions the cold-storage value of cell Na has an influence on the incubation concentration, a large change in the former being associated with a small change in the same direction, of the latter. This is not surprising. The cell has to excrete not only Na constantly diffusing into it from the plasma through passive areas of the cell membrane in amounts depending on the plasma level, but also Na already present in the cell at the beginning of incubation, and the concentration of the latter which has been made to vary between 13 and 84 m.equiv. is by no means negligible. In order to evaluate the influence of cell Na at the beginning of incubation on the value at the end, the ratios of cell- and plasma-Na concentrations, $[I]/[P]$, have been plotted against the Na concentrations of the corresponding cold-stored cells, $[C]$. In Fig. 1, the relation $[I]/[P]$ to $[C]$ is shown for plasmas of high, medium and low Na concentrations. It must be noted that a high degree of accuracy was not possible in fitting curves, partly because it was not practicable to

investigate more than three cold-storage levels, partly through difficulties in reproducing exact pH conditions in each blood sample incubated, and partly through increasing error in the Na method when low contents were estimated. However, the following conclusions seem justified: if one accepts, what is probable, that at equilibrium in an ideal system $[I]/[P]$ is constant, then the degree of obliquity of the curves to the X-axis is an index of the functional activity of the cells, for the less the obliquity, the less will be the influence of cold-storage Na on the value at the end of incubation. Further, if at equilibrium $[I]/[P]$ were constant, then the distance of the corresponding horizontal line from the X-axis would be an index of the balance between cell permeability and speed of passive diffusion on the one hand and activity of output on the other. This indication will also be gained by extrapolating the curves of systems not in equilibrium in Fig. 2, to $[C]=0$, and the values of $[I]/[P]$ so obtained will mainly be an index of cell permeability, for when cell Na falls from a high level against the concentration gradient, the chief factor is active output; when it rises from a low level with the gradient, passive diffusion predominates. In support of this view, it will be noted that in Exps. 1 and 2, where incubated cells were not kept suspended by rocking and where active output of Na produced a local rise of Na round the cells, thus increasing back diffusion, the heights of the curves above the X-axis were also much increased.

But although it is likely that at true equilibrium $[I]/[P]$ would be constant, under experimental conditions this constancy is not attained. Preliminary investigations indicate that cell Na falls sharply for 9–15 hr. and then for the rest of the 24 hr. incubation, alters very little, $[I]$ remaining constant for each individual system, while the various values of $[I]/[P]$ for the constituent systems of each experiment are not quite identical, being still somewhat dependent on $[C]$. It is suggested that several days would be required for $[I]/[P]$ to become constant and that long before this the activating mechanism has begun to fail, so that although after 12 hr. incubation it still suffices to keep cell Na at a constant low level, it is unable to lower the level still further to that point at which $[I]/[P]$ is constant. Such weakening of output activity might be due to decay in the phosphorylation cycle, since after 24 hr. incubation, although glucose is still present in the cell, organic phosphate has fallen from 95 to 30% of the total phosphate.

If, however, at equilibrium $[I]/[P]$ is in fact constant, then $([P]-[I])/[P]$ is also constant, and in Table 2 this relation roughly holds though the experiments there are not presumed to have reached equilibrium. This probably arises because the systems are approaching equilibrium and partly because in the fraction, $[P]$ is relatively great, while $[I]$ is small with variations which, though relatively moderate, are absolutely small. It is probable that fuller study of the dynamics of output (with special reference to Na permeability as measured by trace and to the effects of variation of lithium in the external phase)

would clarify the problem; at present it can be said that $[Na]_{cell}$ after incubation is mainly determined by $[Na]_{plasma}$, but owing to failure of the system to reach equilibrium, is affected to some extent also by $[Na]_{cell}$ at the start of incubation. *In vivo*, however, absence of metabolic failure ensures a constant relation of cell Na to plasma Na, the balance struck between active output and passive diffusion determining individual and species variations in cell Na and cell K.

Effects of plasma-potassium concentration on active sodium output

It has been seen that when erythrocytes are incubated in a medium containing glucose but poor in K, apparent output of Na falls; this is because when external K is not available as a compensating cation, cell anion attracts Na which diffuses back passively into the cells as fast as it is actively excreted. It may be questioned if real output also fails or whether sufficient output persists to overcome in whole or part the tendency for Na to diffuse into the cells, and since in Exp. 2 (Table 3) cell Na rises by 23 m.equiv./l. in the absence of glucose (contents column *a* and *c*), while in the presence of glucose no Na enters (*a* and *g*), it seems likely that even when plasma K is very low and no apparent output of Na is seen, some real output still occurs. However, it was said when considering 'methods' that the glucose-free control is not always valid for various reasons, of which the chief was the divergence of its pH during incubation from that of bloods containing glucose. This objection in the present context is not serious, since the real outputs compared are in each case the difference between values in a glucose-free system whose pH shifts from 7·1 to 7 and in a glucose-containing system with a shift from 8 to 7, so that although each real output may not have an absolute meaning, one has a significance relative to the other. In any case in Exp. 3, pH was similar in all systems owing to the use of buffered plasma. But one difficulty still remains: during the incubation of glucose-free blood the concentration gradient for Na falls throughout incubation, while with glucose present it rises, so that passive back-diffusion in the latter, though not manifest, might well be greater than in the former. On the other hand, it may be that passive return of Na in glucose-containing systems is restricted by the simultaneous inward diffusion of K through the same areas of the cell surface, and that concealed back-diffusion in the presence of glucose does not differ appreciably from simple manifest diffusion in the absence of glucose. If this were so, then in Exp. 3 of Table 3, in addition to the obvious fall in apparent output of Na as external K falls, there would also be a fall in real output, which with high external K was 20 m.equiv. (*b* minus *f*) and with low external K was between 14 and 18 m.equiv. (*e* minus *h* or *i*). So too in Exp. 2, with high and low external K, the respective real outputs were 40 (*b* minus *d*) and 23 m.equiv. (*c* minus *g*). The question arises if in the complete absence of external K, real output would fail altogether. If so, the glucose-free

control would be fully valid because passive diffusion alone would be occurring both in glucose-free and glucose-containing systems. Unfortunately media cannot be made K free, for if cells are washed free of intercellular K and suspended in K-free media, a little K soon enters the medium either by lysis of cells or simple leakage, Thus in Exps. 1 *c*, *h*, 2 *c*, *g* and 3 *e*, *i*, external media were originally K free but acquired a little K by the end of incubation. A solution might be attempted by plotting so-called real output against external K and extrapolating to K = 0, but points on the curve are too few and the lowest values for K too high for this to be possible. However, it seems likely that real output of Na decreases as external K falls, and that though output does not depend on uptake of K by the cells it is in some way potentiated by the presence of K in the plasma; but a conclusive statement cannot be made until passive movement of Na can be measured, while active output is proceeding and this may require the use of radioactive Na.

It does, however, seem quite definite that although apparent output of Na fails when external K is low, total base and volume after incubation are still below the cold-storage levels because there is no uptake or actual loss of K. Thus, in Exp. 1 of Table 3, the cold-storage level of total base is 119 m.equiv./l., while the incubation levels are 110 with high plasma K and 115 with low (1 *a*, *b* and *h*). In Exps. 2 and 3 the findings are similar. Thus the volume and base control mechanism still works effectively even when output of Na seems relatively inactive, so that the ratio of Na/K in the cell does not fall as low as usual after incubation.

pH *and cell cation*

It has been seen earlier that apparent active cation movements are most marked between pH 7·2 and 7·4. It is probable that passive movements in the presence of glucose are qualitatively, if not quantitatively, similar to diffusion in the glucose-free controls. Since the gap between the two curves is widest between pH 7·2 and 7·4, it follows that real output is most active in this range (Fig. 2). But when considering the maintenance of the constancy of cell base *in vivo*, it is the apparent and not the real cation exchange which is effective. It has been seen that this is maximal, or nearly so, over a wide range between 6·85 and 7·65. Normal pH measured by similar methods is 7·2–7·4 at 20°, and it is unlikely that in acidosis or alkalosis cell pH will transgress the range of maximum cation activity. Nevertheless, Butler and his co-workers (1947), investigating diabetes, and Darrow and his associates (1948), in infantile diarrhoea, have found that with severe acidosis, there is a loss of K and water from the body. It must be presumed that the very actively metabolizing tissue cells are more adversely affected by acidosis than are the erythrocytes, and that they lose K in circumstances which leave the red cell almost unaffected.

Control of cation in erythrocytes

It has been seen that major factors in fixing cell base are the concentration gradients sending Na into and K out of the erythrocytes, non-penetrating cell anion attracting all ions and also water, and active output of Na and water removing base from the cell, but also tending to raise the concentration of cell non-penetrating anion and so increase backflow of penetrating ions. Subsidiary factors are the pH of the cells and the levels of plasma Na and K. In man the ratio *in vivo* of $[Na]_{red\ cells}/[Na]_{plasma}$ is about 0·13, and in successful experiments similar ratios may be obtained on incubation *in vitro*. But constancy of cell base and volume may also be found in systems with low plasma K, where the cell-plasma [Na] ratio is high because of increased back-diffusion of Na and cell K low. Such a system would give incubation curves of the type shown in Fig. 1, but placed higher above the base-line, even with rapidly glycolysing and respiring cells. It is possible that the erythrocytes of certain species (ox, dog, cat, etc.) with a high cell-plasma [Na] ratio and low cell K are similar to these experimentally modified human cells in having a powerful active output largely neutralized by an increased passive backflow of Na; the latter due to a low level of plasma K or to high cell permeability. On the other hand, one may conceive of systems in which the cell-plasma [Na] ratio is high not because of raised permeability, but because output is low, in which case constancy of cell base would be achieved with a real saving in work done. Incubation curves from such cells would be more oblique than those in Fig. 1 and cell glycolysis would be small and respiration slight.

However this may be, without some such mechanism for controlling the entry of salts and water into cells incapable of resisting an internal pressure, rupture is inevitable. The mode of activation is still unknown; its dependence on glycolysis is clear and work in hand shows that it is inhibited by fluoride and iodoacetate, but not by carbon-monoxide or cyanide.

SUMMARY

1. When human blood is cold-stored cell Na rises and cell K falls in accordance with the respective concentration gradients. Total base increases.

2. On incubating such cold-stored cells in the presence of glucose, Na leaves the erythrocyte and K enters against the concentration gradients, the value for Na + K falling. The result of these actions in the intact circulation is to prevent that accumulation of salts and water which would otherwise occur because of the excess of cell over plasma non-penetrating anion. Exit of Na is active and uptake of K passive.

3. When red cells take up lithium from an appropriate diluent during cold-storage, this is not actively excreted during incubation.

4. Output of Na is very active between pH 6·85 and 7·65, the actual maximum lying between 7·2 and 7·4. It is unlikely that pathological variations in the

pH of erythrocytes suffice to cause significant changes in the balance of cell Na and cell K, but this does not exclude the possibility of similar pH changes in more actively metabolizing tissue cells causing serious cation disturbance.

5. The level to which cell Na falls on incubation depends chiefly on the plasma-Na concentration and to a less extent on the value of cell Na at the beginning of incubation. Active output and passive backflow balance in man when the red cell-Na concentration is about one-eighth of the plasma concentration.

6. When erythrocytes are incubated in plasma poor in K, output of Na falls.

7. A distinction has been drawn between apparent and real output of Na. The former is the effective output and equals the difference between cell Na at the beginning and end of incubation. Real output includes apparent output together with that output of Na which opposes and overcomes the tendency for Na to enter the erythrocytes by simple diffusion.

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THE INFLUENCE OF CALCIUM, POTASSIUM AND MAGNESIUM IONS IN CEREBROSPINAL FLUID ON VASOMOTOR SYSTEM

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The influence of the ionic concentration of the cerebrospinal fluid on the vasomotor system has been investigated by several authors. Stern & Chvoles (1933) found that the introduction of potassium into the cerebral ventricles of dogs and cats causes an elevation of the arterial pressure, a stimulation of the pressor and an inhibition of the depressor reaction of the carotid sinus reflexes. Calcium, on the other hand, has a reverse effect. Resnik, Mason, Terry, Pilcher & Harrison (1936) observed that potassium, injected intracisternally into dogs, lowers the arterial pressure in slight concentrations and raises it in greater concentrations. Intracisternally injected, calcium causes little or no change in blood pressure and has no influence on the carotid sinus reflexes. However, it strongly counteracts the action of potassium. The same results are obtained with magnesium, though less intensively. Von Euler (1938) obtained an elevation of the arterial pressure in the cat by the intracisternal injection of KCl. He found in his experiments that calcium had little effect, although it neutralized the action of potassium. According to Mullin, Hastings & Lees (1938) a rise in arterial pressure was noted by increasing the potassium or lowering the calcium concentration in the cerebrospinal fluid of dogs. Downmann & Mackenzie (1943) reported that potassium, intracisternally injected, causes an elevation of blood pressure in rabbits. Sometimes the elevation is preceded by a slight depression. The same observations were made by Walker, Smolik & Gilson (1945) in dogs. No clear results were obtained by these investigators with calcium. The technique employed in their experiments was the intraventricular or intracisternal injection. The disadvantages of this method are that during the injection variations in the cerebrospinal fluid pressure are caused, that the concentration of injected salts may differ widely in the different ventricles and that these concentrations are unknown factors. Further, it is impossible, using this technique, to remove ions from the ventricles

and therefore to study the influence of a decrease of concentration of specific ions. Since the results obtained so far, using this method, do not tally and are incomplete, we have studied the problem using another method which has not the same disadvantages, namely the perfusion of the cerebral ventricles in dogs.

METHODS

Experiments were performed on 48 dogs, anaesthetized with morphine-chloralosane. To study the vasomotor reflexes, the carotid sinuses were isolated from the circulation by means of a technique which is slightly different from that used formerly by Moissejeff (1927), Koch (1931) Heymans, Bouckaert & Regniers (1933) and others. The internal carotid artery is ligated above the carotid sinus and the external carotid artery at its origin. In this way the communication between the chemoreceptors of the carotid bifurcation, situated in the glomus caroticum (supplied by the occipital artery) and the common carotid artery is severed but the pressoreceptors, situated in the carotid sinus, remain in connexion with the common carotid artery. The common carotid artery is ligated about 5 cm. below its bifurcation and a cannula inserted. The blood remaining within the sinus is heparinized. The cannula is attached to a pressure bottle containing Ringer solution and the pressoreceptors can be exposed to any selected pressure, the actual pressure being recorded by an Hg manometer. By changing the pressure repeatedly in the same manner during the experiment, it is possible to elicit the carotid sinus reflexes at regular intervals and to examine the changes produced in it by different factors. The two vagus nerves in the neck are cut in order to exclude the interference of the cardioaortic regulation mechanism with the evoked carotid sinus reflexes. The vasomotor reflexes are also studied by faradic stimulation of the proximal end of the vagus nerve in the neck. The arterial pressure in the femoral artery is registered by means of a mercury manometer. After preparation of the dog in this way, the head is flexed on the body to allow the insertion of the perfusion needles. The first needle is inserted through a small trephine opening in the skull into a lateral ventricle; a second needle is introduced suboccipitally into the cisterna. It is thus possible to perfuse the cerebral ventricles with an artificial solution, the fluid entering through the first needle and leaving through the second.

Perfusion installation

The outlets of several Mariotte bottles, each containing a different perfusion fluid, are all connected to one tube which passes through a thermostatted water-bath maintained at body temperature and to the needle placed in the lateral ventricle. Any one of the solutions can be perfused at will and the perfusion pressure, maintained within normal cerebrospinal fluid pressure limits, is measured by means of a water manometer.

Perfusion solutions

The following balanced isotonic solution, approximately the composition of normal cerebrospinal fluid, was used as a basic solution in our experiments. This solution (solution I) has been previously used by Merlis (1940).

	g./l.		g./l.
NaCl	8·10	$NaHCO_3$	1·76
KCl	0·25	NaH_2PO_4	0·07
$CaCl_2$	0·14	$(NH_2)_2CO$	0·13
$MgCl_2$	0·11	Glucose	0·61

In the other solutions the KCl, $CaCl_2$ or $MgCl_2$ concentration was varied, the other salts remaining in the original concentrations with the exception of NaCl which was increased or diminished in order to maintain the isotonicity of these solutions. Their composition was as follows.

High calcium. Solution II*a*: 1·00 g. $CaCl_2$, 7·42 g. NaCl—as solution I. Solution II*b*: 2 g. $CaCl_2$, 6·67 g. NaCl—as solution I. Solution II*c*: 0·28 g. $CaCl_2$, 7·99 g. NaCl—as solution I.

Low calcium. Solution III*a*: 0·00 g. $CaCl_2$, 8·21 g. NaCl—as solution I. Solution III*b*: 0·07 g. $CaCl_2$, 8·15 g. NaCl—as solution I.

High potassium. Solution IV*a*: 0·50 g. KCl, 7·91 g. NaCl—as solution I. Solution IV*b*: 1·00 g. KCl, 7·51 g. NaCl—as solution I. Solution IV*c*: 5·00 g. KCl, 4·26 g. NaCl—as solution I.

Low potassium. Solution V: 0·00 g. KCl, 8·29 g. NaCl—as solution I.

High magnesium. Solution VI: 1·00 g. $MgCl_2$, 7·28 g. NaCl—as solution I.

Low magnesium. Solution VII: 0·00 g. $MgCl_2$, 8·20 g. NaCl—as solution I.

The pH of all solutions was adjusted to 7·4 by bubbling carbon dioxide through them.

The use of this technique has the following advantages:

(1) There is no pressure change when a solution with an abnormal ion concentration is perfused.

(2) All the centres lying in relation to the ventricles come into contact with the perfusate and therefore under the influence of the same known ionic concentration.

(3) It is possible to eliminate almost completely any particular ion out of the cerebral ventricles.

(4) One is able to observe by returning to the perfusion with the basic solution whether the action of the ionic variation is reversible.

Description of an experiment

Beginning with the basic solution, the carotid sinus reflexes (pressor and depressor reaction) were regularly evoked by changing at regular intervals and, similarly, the pressure in the isolated carotid bifurcation. When reflex hypertension was produced by faradic stimulation of the proximal end of the vagus in the neck, the intensity and the duration of the stimulus were regulated to obtain a pronounced reaction. This stimulation was regularly repeated throughout the experiment. Other solutions were consecutively substituted for the basic solution. Thus variations in reflex activity of the vasomotor system caused by perfusion of solutions with abnormal ionic concentrations were studied. Perfusion in each case was continued until the arterial pressure and the vasomotor reflexes were stablized. When a solution other than the basic solution was being used, the perfusion of the abnormal solution was discontinued after its effect had been ascertained, and it was replaced by that of the basic solution to see if the effect was reversible.

RESULTS

Perfusion with the basic solution (solution I).

In order to determine whether or not the vasomotor tone and reflexes remain unchanged under the influence of a perfusion with basic solution, we compared the blood pressure and the vasomotor reflexes present after vagotomy and carotid sinus isolation, but before the insertion of the perfusion needles with those existing during the perfusion with the basic solution. The results show that no substantial differences were observed (Figs. 1 and 2). Further, it was noted that the blood pressure and the vasomotor reflexes remained constant as long as the basic solution was perfused. Taking these values as a standard, it was possible to determine what deviation from the normal was brought about by alteration in the ionic concentrations of the perfusion fluid.

Influence of the calcium ion concentration

The influence of an increased calcium concentration was studied by perfusing the cerebral ventricles of fourteen dogs with solutions containing a high $CaCl_2$ content. These solutions lowered the arterial pressure, depressed the carotid sinus reflexes (pressor and depressor reactions) and lowered the hypertension caused by faradic stimulation of the proximal end of the vagus nerve (Figs. 3 and 4). These results were obtained when the $CaCl_2$ concentration was increased

to 14, 7 and 2 times the normal concentration (solutions II*a*, II*b* and II*c*). This action was reversible.

The effect of lowering the $CaCl_2$ content was investigated in nine experiments in which the cerebral ventricles were perfused with a solution containing no calcium ions (solution III*a*). The arterial pressure showed a marked rise and

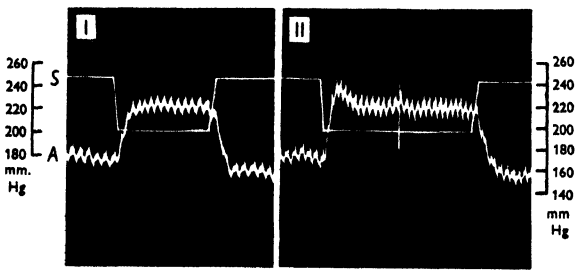

Fig. 1. Dog, 12·5 kg. Morphine-chloralosane anaesthesia; bivagotomy; isolated carotid sinuses. *S*, pressure in the carotid sinuses; *A*, arterial pressure. Arterial pressure and carotid sinus reflexes before (I) and during (II) perfusion with the basic solution. No remarkable change in blood pressure and reflexes.

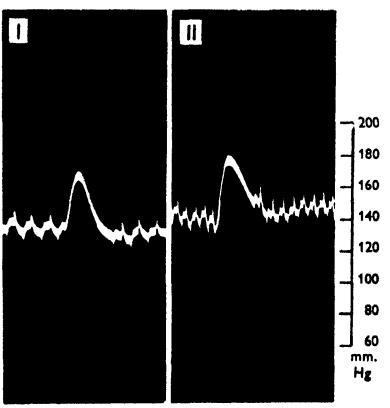

Fig. 2. Dog, 7·5 kg. Morphine-chloralosane anaesthesia; bivagotomy; isolated carotid sinuses. Arterial pressure and reflex hypertension on faradic stimulation of the proximal vagus end in the neck before (I) and during (II) perfusion with the basic solution. No remarkable change in blood pressure and reflex hypertension.

the vasomotor reflexes were generally more pronounced than under normal conditions (Fig. 5). Even when perfusing with a solution reduced to half the normal $CaCl_2$ concentration (solution III*b*), this effect was obvious. In some of the experiments, however, a rise of the arterial pressure was noted, but no increase of the vasomotor reflexes. This was usually observed when the arterial pressure reached a very high level. This is probably not due to a depressed reflex activity of the vasomotor system, but rather to the original high arterial

pressure, which allows only a slight further increase when the pressure in the carotid sinus is lowered or when the proximal end of the vagus is stimulated. On returning to the perfusion with the basic solution, the blood pressure and the vasomotor reflexes returned to normal.

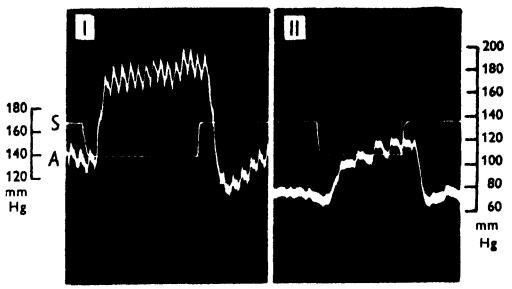

Fig. 3. Dog, 13·5 kg. Morphine-chloralosane anaesthesia; bivagotomy; isolated carotid sinuses. *A*, arterial pressure; *S*, pressure in the carotid sinuses. Arterial pressure and carotid sinus reflexes during perfusion of the cerebral ventricles with the basic solution (I) and with a calcium-rich solution (II) (1 g. $CaCl_2$/l.; solution II). The arterial pressure and carotid sinus reflexes are markedly depressed during the perfusion with the calcium-rich solution.

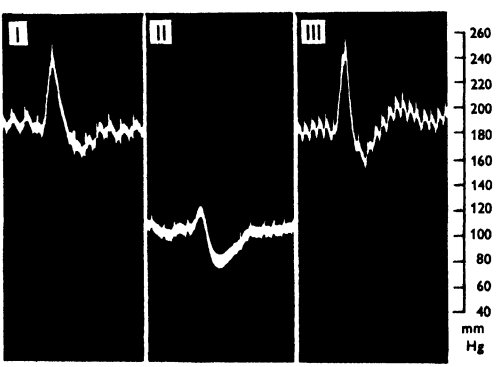

Fig. 4. Dog, 11 kg. Morphine-chloralosane anaesthesia; bivagotomy; isolated carotid sinuses. Reflex hypertension on faradic stimulation of the proximal vagus end in the neck. I, perfusion with the basic solution; II, perfusion with a calcium-rich solution (1 g. $CaCl_2$/l.; solution II); III, subsequent perfusion with the basic solution. Blood pressure and reflex are strongly depressed during perfusion with the calcium-rich solution.

Influence of the potassium ion concentration

Proceeding from the perfusion with the basic solution to that with a solution of increased KCl concentration (solution IV*a*, IV*b* and IV*c*), a marked increase in arterial pressure and vasomotor reflexes was noted in ten dogs (Fig. 6). On perfusing with solution IV*c*, a marked blood pressure rise of about 180 mm. Hg rapidly developed. With solution IV*b* the arterial pressure rise varied between

20 and 110 mm. Hg with an average of 50 mm. Hg. With a solution containing twice the normal KCl concentration (solution IV*a*) there was still a rise but less marked (30–40 mm. Hg).

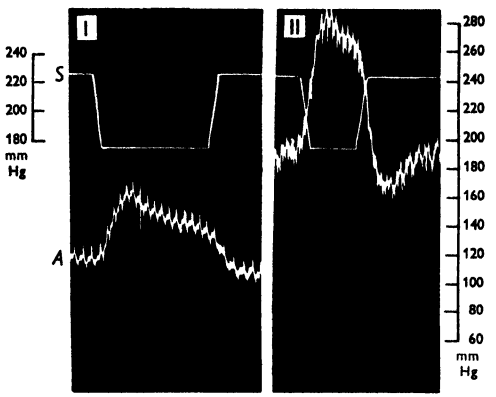

Fig. 5. Dog, 7·5 kg. Morphine-chloralosane anaesthesia; bivagotomy; isolated carotid sinuses. *A*, arterial pressure; *S*, pressure in the carotid sinuses. Arterial pressure and carotid sinus reflexes during perfusion of the cerebral ventricles with basic solution (I) and with a calcium-free solution (II) (solution III). Marked rise in arterial pressure and reflex intensity during perfusion with the calcium-free solution.

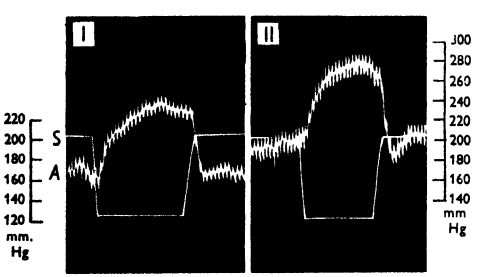

Fig. 6. Dog, 17·5 kg. Morphine-chloralosane anaesthesia; bivagotomy; isolated carotid sinuses. *A*, arterial pressure; *S*, pressure in the carotid sinuses. Carotid sinus reflexes during perfusion of the cerebral ventricles with basic solution (I) and with a potassium-rich solution (II) (1 g. KCl/l.; solution IV*b*). Rise in arterial pressure and reflex intensity during perfusion with the potassium-rich solution.

The carotid sinus reflexes were more pronounced during the perfusion with potassium-rich solutions than during that with basic solution. However, this enhancement of the reflex activity of the vasomotor system was more clearly observed when the blood pressure had not reached too high a level. This was particularly the case when there was only a slight increase in KCl. When perfusing with a solution of high KCl concentration, the rise of the blood pressure was so pronounced that only a slight further rise could be obtained by evoking the vasomotor reflexes.

The hypertension caused by faradic stimulation of the proximal end of one vagus nerve was also more marked during the perfusion with a potassium-rich solution than during that with the basic solution. The same exception as above must be made however when a very high arterial pressure was present. On returning to the perfusion with basic solution, the arterial pressure and the vasomotor reflexes became normal.

During the perfusion with the potassium-free solution (solution V) no marked changes were observed in the blood pressure and in the vasomotor reflexes. In one of our experiments, a slight depression of the arterial pressure (5 mm.Hg) was noted, in three a little rise (20, 10 and 6 mm. Hg), while in the other experiments (ten) no change occurred. The reflexes also remained constantly the same. In one experiment we perfused the cerebral ventricles with the potassium-free solution for 3 hr. without effect.

Mechanism of the central action of calcium and potassium on the vasomotor system

An attempt was made to analyse the mechanism by which the lowering of calcium and the increase of potassium in the cerebral ventricles cause an elevation of blood pressure. Using the technique of Nolf (1902) with the three manometers, alterations in the vasomotor tone in the peripheral circulation under these conditions was investigated first.

The method is as follows. In dogs, anaesthetized with morphine-chloralosane and bivagotomized, the carotid sinuses were isolated and the cerebral ventricles perfused as described above. The arterial pressure was recorded with a mercury manometer which was connected to the proximal end of one femoral artery. The pressure reading was also taken on the peripheral ends of the two femoral arteries, one of the hindlegs having been denervated, the other one remaining intact.

On perfusion with the high potassium or low calcium solutions, the arterial pressure rose as previously described. In the intact hindleg, the pressure in the peripheral end of the femoral artery increased rapidly, while in the denervated hindleg, the pressure rose more gradually and did not reach the same level. It was concluded that the peripheral vasomotor tone increases under these conditions. It might be suggested that the rise of pressure in the intact leg is not due to an increase in the vasomotor tone but rather is caused by an increase in the skeletal muscle tone. To examine this hypothesis, similar experiments were performed after curarization of the dogs so that all the skeletal muscles were paralysed, the animals being kept alive by means of artificial respiration. The effect was found to be the same as in the former experiments.

In further experiments, changes in the splanchnic area under similar conditions of ventricular perfusion were examined, the volume of the spleen being recorded for this purpose. It was noted that the spleen contracted when the potassium concentration was increased or the calcium concentration lowered in the fluid perfused through the cerebral ventricles. This led to the assumption that these factors caused vasoconstriction in the splanchnic area. This constriction, however,

can be evoked by nervous influence or by increased adrenaline production. Observing the reactions even after denervation of the spleen, it appeared that constriction in the splanchnic area is, at least partly, caused by increased adrenaline liberation. On the other hand, we found that the constriction of the normally innervated spleen still occurred after bilateral adrenalectomy. As a result of these experiments, it was concluded that the constriction in the vessels is partly due to an increased secretion of adrenaline and partly related to a nervous influence. These results are in agreement with the conclusions of de Vleeschhouwer (1935) and Hermann, Jourdan, Morin & Vial (1938). Since curarization of the dogs did not prevent the circulatory effects, it seems that skeletal muscular activity is not as important as was suggested by Mullin *et al.* (1938).

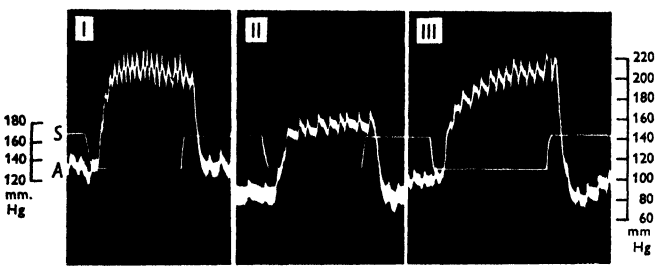

Fig. 7. Dog, 13·5 kg. Morphine-chloralosane anaesthesia; bivagotomy; isolated carotid sinuses. *A*, arterial pressure; *S*, pressure in the carotid sinuses. Arterial pressure and carotid sinus reflexes during: I, perfusion with basic solution; II, perfusion with a magnesium-rich solution (1 g. $MgCl_2$/l.; solution VI); III, subsequent perfusion with basic solution. Depression of arterial pressure and carotid sinus reflex during perfusion with the magnesium-rich solution.

Influence of the magnesium ion concentration

In eight experiments the arterial blood pressure fell when the ventricles were perfused with a solution of increased $MgCl_2$ concentration (solution VI). Also the carotid sinus reflexes and the hypertension caused by faradic stimulation of the proximal end of the vagus were depressed (Fig. 7). This action was very similar to that of $CaCl_2$, but using the same excesses of both ions, the action of calcium was more pronounced.

Little or no changes occurred in the arterial pressure and vasomotor reflexes in ten experiments in which the perfusion fluid contained no magnesium (solution VII). Only in one experiment was an increase of the carotid sinus reflexes noted.

DISCUSSION

Calcium. A depression of the arterial pressure under influence of an increased calcium concentration was constantly observed in the experiment. This corresponds with the observations of Stern & Chvoles (1933) and does not confirm von Euler (1938), Resnik *et al.* (1936) and Walker *et al.* (1945) who noted

little or no depression of the blood pressure by this influence. Even when the normal $CaCl_2$ concentration was only twice the normal, the effect was evident. It must be remembered, however, that the experiments were carried out after vagotomy and carotid sinus isolation. So the reflex mechanism by which the body is able to maintain a steady arterial pressure in the presence of factors which tend to vary that pressure, was put out of action. Thus blood-pressure changes were more marked and more easily observed than in a normal animal. The observations of Resnik *et al.* (1936) that calcium has no influence on the carotid sinus reflex were not confirmed in our experiments.

The rise of the arterial pressure, observed when the calcium content in the cerebrospinal fluid is lowered, is in agreement with the results of Mullin *et al.* (1938) and Huggins & Hastings (1933). This influence was still clear when the $CaCl_2$ content of the perfusion fluid was reduced to half of the normal. The carotid sinus reflexes were enhanced and the hypertension produced by faradic stimulation of the proximal end of the vagus in the neck was increased. There was also a simultaneous respiratory and muscular stimulation.

The influence of the increase and decrease of the $CaCl_2$ concentration was completely reversible, and on perfusing once again with the basic solution, the blood pressure and the vasomotor reflexes returned to normal.

Potassium. In agreement with the authors mentioned in the introduction we observed a rise of arterial pressure on increasing the potassium concentration in the cerebral ventricles. This rise was obtained as well by a relatively slight increase of the KCl concentration in the perfusate (twice the normal strength) as by a much greater increase (4 to 20 times the normal). Using small KCl excesses the blood pressure rose slowly and reached a level which was maintained as long as the perfusion was continued. These results differ thus in important respects from the circulatory reflexes obtained by Calma & Wright (1947) by injecting K^+ into the lower spinal theca. Walker *et al.* (1945) are of the opinion that potassium stimulates the sympathetic and the parasympathetic centres. Stern & Chvoles (1933), on the contrary, believe that the sympathetic centres are stimulated while the parasympathetic are depressed. This was concluded from the fact that they observed a stimulation of the pressor reaction of the carotid sinus and an inhibition of the depressor reaction. In our experiments, however, the depressor reaction as well as the pressor reflexes were stimulated. Further, the stimulation included the respiration and the muscular tone. This confirms, in our opinion, the statement of Walker *et al.* that potassium exerts a general stimulation on the central nervous system.

No experiments are known to us concerning the influence of lowering the potassium concentration in the cerebrospinal fluid. In a review (1945) about the Ca/K balance in the cerebrospinal fluid, Stern, Moguilnitsky, Schattenstein & Kassil believe that the lowering of the potassium concentration acts in the

same way as the increase of the calcium concentration. According to our experiments a potassium-free solution has no effect, the arterial pressure and the vasomotor reflexes remaining the same as during the perfusion with the basic solution. Perhaps this is somewhat difficult to explain, since potassium ions are known to have marked effects on the nervous system. However, potassium is chiefly an intracellular ion and passes out with great difficulty, as was observed by Fenn, Cobb, Hegnauer & Marsh (1934), the potassium ions being bound to large organic anions, which cannot diffuse through the cell membrane. Schmidt & Bear (1939) obtained identical results in the axoplasm of squid giant. Our negative results are probably due to the fact that no substantial alterations in the potassium content of the nerve cells occurred during the perfusion, which lasted generally 30 min., excepting in one case where the perfusion time was 3 hr. According to Fenn (1940) nerves brought into contact with an isotonic sugar solution retained 60% of their original potassium content after 12 hr. immersion.

Magnesium. In accordance with the results of Resnik *et al.* (1936) we found that a high magnesium concentration in the cerebral ventricles produced similar effects to a calcium excess, though with less intensity. This is contrary to the action of both ions in the blood, for Bryant, Lehmann & Knoefel (1939) found an antagonistic action between Mg and Ca in the blood on the central nervous system as did Heymans & Capet (1945) for the vasomotor carotid sinus reflexes.

On lowering the magnesium concentration in the cerebral ventricles little or no influence on the vasomotor system was noted. In the literature also we found no publications about this problem.

SUMMARY

1. The perfusion of the cerebral ventricles in dogs, after bivagotomy and carotid sinus isolation, with an isotonic solution of a similar composition to the normal cerebrospinal fluid causes no great change in the blood pressure. The carotid sinus reflexes and the reflex hypertension on faradic stimulation of the proximal end of vagus in the neck are unchanged.

2. An excess of $CaCl_2$ in the perfusate causes a depression of the blood pressure and of the studied vasomotor reflexes.

3. A lowering of the calcium concentration in the cerebral ventricles raises the arterial pressure and enhances the vasomotor reflexes.

4. An excess of potassium in the perfusate of the cerebral ventricles raises the blood pressure and enhances the vasomotor reflexes.

5. The perfusion of the cerebral ventricles with a potassium-free solution has no effect on the arterial pressure and the vasomotor reflexes.

6. The blood-pressure rise, caused by diminishing the $CaCl_2$ concentration or by augmenting the KCl concentration in the perfusate, is produced by several

mechanisms. The vasomotor tone increases in these conditions in the peripheral vessels and in the splanchnic area. There is also an increased adrenaline secretion.

7. An excess of magnesium in the perfusate of the cerebral ventricles causes a depression of the arterial pressure and of the vasomotor reflexes.

8. The perfusion of the cerebral ventricles with a magnesium-free solution causes little or no changes in the arterial pressure and the vasomotor reflexes.

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ACCLIMATIZATION TO HEAT AND COLD

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A fall of the rectal temperature, the skin temperature and the pulse rate can be detected in human subjects after a few days, or even a number of hours, in a hot environment, and this was considered to be a sign of acclimatization to heat (Bean & Eichna, 1943; Robinson, Turrell, Belding & Horvath, 1943; Eichna, Bean, Ashe & Nelson, 1945), but definite signs of acclimatization to cold were not obtained after repeated or continued experimental exposures to low environmental temperatures (Adolph & Molnar, 1946; Horvath, Freedman & Golden, 1947; Stein, Eliot & Bader, 1949), although a few authors have described minor signs of adaptation (Scott, Bazett & Mackie, 1940; Glickman, Keeton, Mitchell & Fahnenstock, 1946). The present investigation was planned to show whether acclimatization to cold did exist, and it also offered an opportunity of studying two further problems. (1) How were the movements of blood between the inside of the body and the extremities which were shown to accompany changes of the environmental temperature (Glaser, 1949*b*) affected by continued exposures to heat and cold? (2) Was acclimatization to heat and, if its existence could be demonstrated, acclimatization to cold modified by frequent changes of environmental temperature?

PROCEDURE

Six intelligent and healthy sailors aged 19–20 years had volunteered for the experiment. All had been experimental subjects before, but none of them had been exposed to any extremes of climate for 2 months before the present investigation began. In the cold-room they wore thin woollen underwear, flannel shirts, sleeveless woollen pullovers, serge or flannel trousers, tweed or leather jackets, woollen socks, and leather slippers or shoes. In the hot-room they wore canvas shoes and linen shorts. At night they slept in flannel pyjamas on beds with horsehair mattresses. They used 4 woollen blankets in the cold-room.

The subjects were tested in two groups of three. Each group spent three successive 72 hr. periods alternately in a hot and cold environment, group I starting in the cold-room and group II in the hot-room. Each period began and ended at 10 a.m. Measurements were taken at 3 p.m., 8 p.m. and 8 a.m. and took about 1 hr. The former were taken 2 hr. after the end of a meal, the latter before breakfast. The subjects sat still for ½ hr. before each set of measurements with their left arm supported horizontally. Measurements were always taken in the same order with the

subjects sitting still. The subjects only left the air-conditioned rooms twice daily, for 15 min. after breakfast, and for a few minutes before turning in at night. They emptied their bowels and washed at these times. It was intended to keep them fit during the tests without exercising them too hard, and they were, therefore, encouraged to move about as much as possible (except before measurements), and to do gymnastic exercises for 30–45 min. daily. They were given as much food and water as they liked.

The rectal temperature, the skin temperature, the volume of the left forearm and hand, and the vital capacity were measured as previously described (Glaser, 1949*a*, *b*). The skin temperature was measured in seven marked points: over the forehead, the manubrium sterni, the left forearm, the ball of the left thumb, the tip of the left middle finger, the middle of the left thigh, and the middle of the left leg near the anterior edge of the tibia. Since this investigation did not set out to measure the true average surface temperature, representative areas likely to show varying degrees of fluctuation were selected. In the cold-room part of the skin was covered, but it seemed right to measure the temperature both of clothed and exposed areas, which conforms with the principle adopted by Gagge, Winslow & Herrington (1938). The blood pressure was measured with a mercury manometer by auscultation of the brachial artery. The latter was palpated and marked. This and other markings, notably the line used in measurements of the forearm and hand volume (Glaser, 1949*b*), and those used in skin temperature measurements, were kept clear and sharp. The pulse-rate was counted over 30 sec. The urine was collected under toluene in large bottles. While the subjects were in the hot-room the urinary chloride output was estimated every 24 hr. to make sure that the intake of NaCl was adequate to cover all the body's requirements.

The cold-room was adjusted to maintain a temperature of $-1°$ C., but its refrigerating capacity was insufficient and its temperature rose slowly during the day, reaching $+3°$ C., or exceptionally even $+4°$ C., in the evenings. During the night it cooled down again to $-1°$ C. Air movement in the cold-room was negligible. During the first part of the experiment the hot-room was at 40·5° C. (dry bulb) and 35° C. (wet bulb) with an air-movement of 100 ft./min. This corresponds to an 'effective temperature' of 35·5° C. (Bedford, 1946). The plant did not stand up well to continuous running at such a high temperature and during the second part of the experiment, while group II was being tested, the hot-room was kept at 35° C. (dry bulb) and 29° C. (wet bulb) with an air movement of 100 ft./min. This corresponds to an 'effective temperature' of 30° C. In spite of this lowering of the hot-room temperature there was a short breakdown on the second morning of group II's second period in the hot-room; the subjects were kept warm by blankets, but one set of measurements was thus missed. At night the air movement in the hot-room was stepped up to 300 ft./min.

RESULTS

Average results are given to the nearest significant decimal. A difference between two means was considered significant if it amounted to more than twice the standard error of the difference (Bradford Hill, 1942).

Subjective effects

There was some uniformity in the personal impressions recorded in the subjects' diaries. The last day of each period of exposure always seemed to be the least unpleasant, which appears to be evidence of acclimatization to both heat and cold. Moreover, the second exposure to either environmental extreme was always tolerated better than the first. During the last 2 days of the experiment none of the subjects in group I ever shivered or felt uncomfortably cold; and none of the subjects in group II sweated while sitting still, or felt uncomfortably hot.

General results

Table I shows that the average vital capacity and the average forearm and hand volume of both groups were significantly smaller in the cold-room than

TABLE 1. Mean values of all observations in the cold-room and hot-room

		All observations in the cold-room				All observations in the hot-room					
	Group	No.	Mean	S.D.	Error of mean	No.	Mean	S.D.	Error of mean	Difference between the means	S.E. of the difference
Vital capacity (l.)	1	162	4·70	0·565	0·044	81	4·86	0·592	0·064	0·16	0·0793
	2	81	5·12	0·322	0·036	153	5·36	0·366	0·030	0·24	0·0464
Forearm and hand volume (l.)	1	54	1·46	0·020	0·0027	27	1·55	0·089	0·0173	0·09	0·0171
	2	27	1·56	0·115	0·0222	51	1·63	0·095	0·0133	0·07	0·0258
Skin temp. (° C.)	1	378	25·7	6·295	0·324	189	36·2	2·057	0·15	10·5	0·356
	2	189	26·7	7·231	0·530	357	34·7	2·056	0·11	8·0	0·537
Rectal temp. (° C.)	1	54	37·2	0·549	0·075	27	37·8	0·600	0·115	0·6	0·138
	2	27	37·0	0·386	0·074	51	37·6	0·371	0·052	0·6	0·090
Pulse rate	1	54	59	9·05	1·23	27	96	20·54	3·96	37	4·14
	2	27	70	9·64	1·85	50	78	9·59	1·35	8	2·30
Syst. B.P. (mm. Hg)	1	42	127	8·06	1·24	27	117	6·00	1·17	10	1·69
	2	27	133	16·30	3·14	51	117	7·94	1·11	16	3·32
Diast. B.P. (mm. Hg)	1	42	66	11·66	1·80	27	50	17·37	3·34	16	3·80
	2	27	60	14·79	2·85	51	52	19·89	2·76	8	3·98

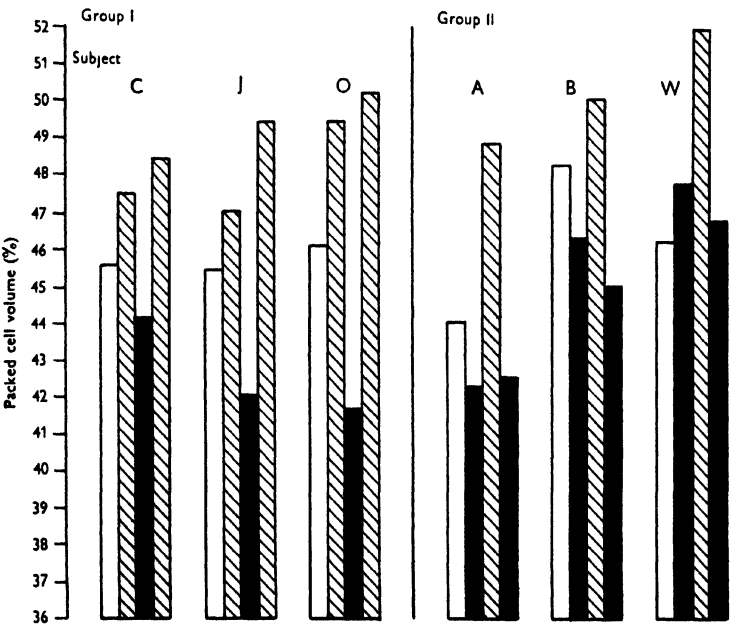

Fig. 1. The packed cell volume before the tests and at the end of each exposure to heat and cold.
Initial level. After 72 hr. in cold. After 72 hr. in heat.

in the hot-room, and this conforms with the results obtained during shorter exposures to heat and cold (Glaser, 1949*b*). Table 1 also shows that the skin temperature, the rectal temperature, and the pulse rate were significantly

lower, whereas the systolic and diastolic blood pressure was significantly higher in the cold-room. Fig. 1 shows that all subjects showed an increase of the packed cell volume (haematocrit ratio) during each period of cooling and a decrease during each period of warming. The erythrocyte count and haemoglobin level behaved similarly, but estimations of the white blood cell count and differential blood-counts produced no relevant data.

Effects of prolonged exposures

Tables 2 and 3 give the mean differences between comparable readings taken from the same subject at the same time of the day (and in Table 2 also from the same spot of skin), during the first and third 24 hr. period in the cold-room.

TABLE 2. A comparison of the skin temperature on the first and third day of the first exposure to cold

(Differences between the temperature of identical points on the skin, measured at the same time of the day.)

Subject	Group	No. of obs. each day	Mean difference between corresponding obs. (° C.)	S.D.	S.E. of mean
A	2	21	+0·4	1·90	0·415
B	2	21	+0·4	2·37	0·517
C	1	21	+0·8	1·69	0·369
J	1	21	+0·9	2·57	0·561
O	1	21	+1·0	1·65	0·360
W	2	21	+0·4	2·30	0·502
All subjects		126	+0·65	2·14	0·192

TABLE 3. The difference between comparable measurements of the rectal temperature on the first and third day of the first exposure to cold

Subject	Group	No. of obs. each day	Mean difference between corresponding obs.
A	2	3	+0·2
B	2	3	+0·5
C	1	3	+0·5
J	1	3	+0·6
O	1	3	+0·1
W	2	3	0·0
Total		18	+0·32
		S.D.	0·359
		S.E. of mean	0·0847
			$t=3·782$
			$P<0·01$

Since the subjects of group I spent two 72 hr. periods in the cold, only the first of these exposures was taken into consideration. On the third day in the cold-room all subjects showed a mean increase of the skin temperature and five subjects a mean increase of the rectal temperature. These differences were statistically significant, and it seems reasonable to assume that they were signs of acclimatization to cold. During the third day in the hot-room the average skin temperature of all subjects was 0·9–2·0° C. lower, and the average rectal

temperature of five subjects 0·1–1·1° C. lower than on the first day, which conforms with previous observations on acclimatization to heat. While the subjects remained in the same air-conditioned room, the vital capacity and the volume of the forearm and hand did not fluctuate much from day to day, and the pulse-rate and blood pressure behaved inconsistently.

Effects of alternating exposures

Those adaptations which could be detected towards the end of the first period of cooling or warming generally became more obvious during the second period of exposure to the same medium. Thus the mean skin temperature of all subjects in group I was significantly higher during the second than during the first period in the cold-room, and the mean skin temperature of group II was significantly lower during the second than during the first period in the hot-room (Table 4). All subjects in group I had a greater packed cell volume

TABLE 4. A comparison of the skin temperature during the first and second exposure to the same medium

(Differences between the temperature of identical points on the skin, measured at the same time of the day on corresponding days.)

Group 1. Two 72 hr. exposures to cold, with an intervening 72 hr. period in the hot-room.

Subject	No. of obs. during each 72 hr. period in the cold	Mean difference between corresponding obs. (° C.)	S.D.	S.E. of mean
C	63	+1·0	1·99	0·26
J	63	+1·7	2·56	0·32
O	63	+0·6	2·36	0·30
All subjects	189	+1·1	2·29	0·17

Group 2. Two 72 hr. exposures to heat, with an intervening 72 hr. period in the cold-room.

Subject	No. of obs. during each 72 hr. period in the heat	Mean difference between corresponding obs. (° C.)	S.D.	S.E. of mean
A	56	−0·5	1·38	0·18
B	56	−0·2	2·25	0·29
W	56	−0·7	1·50	0·22
All subjects	168	−0·5	1·77	0·14

at the end of the second period of cooling, and two subjects in group II had a lower packed cell volume at the end of the second period of warming (Fig. 1). All three subjects of group I also had a higher average rectal temperature by 0·2–0·3° C. during the second period in the cold-room, but this was not statistically significant. Moreover, the average vital capacity of group I was 0·09 l. lower, and the average forearm and hand volume 0·1 l. lower during the second exposure to cold, while the average vital capacity of group II was 0·6 l. higher and the average forearm and hand volume 0·01 l. higher during the second exposure to heat. The latter differences were, again, not statistically significant.

DISCUSSION

Although the statistical calculations presented above are only valid for the subjects of the present investigation, they allow conclusions about the adaptive mechanisms which may function following changes of the environmental temperature.

Acclimatization

It was previously suggested that movements of blood between the surface of the body and the inner regions may be an important mechanism of adjustment to changes of environmental temperature (Glaser, 1949*b*). The present investigation suggests that acclimatization perpetuates these movements and that changes of the circulating blood volume play an important part in the latter process.

The findings that the proportion of plasma in the blood was invariably greater in the hot-room than in the cold-room (Fig. 1) conforms with the observations of Barcroft, Meakins, Davies, Scott & Feller (1923), Bazett, Sunderman, Doupe & Scott (1940), Spealman, Newton & Post (1947) and Stein *et al.* (1949) who found that the human body contained a greater volume of blood in a hot environment than in a cool one. In the present investigation the decrease of the total amount of blood after 3 days in the cold-room appears to have been accompanied by a more rapid flow of blood through the body surface, while the amount of blood in the latter remained small. This may explain why the skin temperature of all subjects was higher on the third day of exposure than the first. Conversely, the fall of the skin temperature which was noted after 3 days in the hot-room may have been caused by a slowing down of the blood flow through the small vessels of the skin while these vessels remained well filled, thus bringing about a decrease of the skin temperature. This explanation is at variance with the findings of Scott *et al.* (1940), who noted that the rate of blood flow through the finger continued to increase for several days in a hot-room. It cannot be excluded that changes of the B.M.R. (Burton, Scott, McGlone & Bazett, 1940; Butson, 1949) may have helped to produce some of the effects of acclimatization.

The discrepancy between the results of the present investigation and the findings of Adolph & Molnar, (1946), Horvath *et al.* (1947), and Stein *et al.* (1949) may have been due to the fact that these authors made their subjects work hard or shiver to exhaustion during the exposures to low temperatures. They also stated (Horvath *et al.* 1947; Stein *et al.* 1949) or inferred (Adolph & Molnar, 1946) that such muscular activity was accompanied by a rise of the skin temperature, and it is known that working muscles need more blood than resting ones (Anrep & von Saalfeld, 1935). Exercise was thus accompanied by an increase of the peripheral blood flow which could have been compensated by a decrease of the blood flow through the vascular regions contained

inside the body cavities, and it may have caused changes which were the opposite of those following upon exposure to cold but similar to those caused by exposure to heat. Exercise might, therefore, be expected to inhibit acclimatization to cold and facilitate acclimatization to heat. Indeed, the latter effect was demonstrated by Bean & Eichna (1943). This aspect of acclimatization may be a matter of fine balance between the opening up and closing down of vascular pools, changes of the total amount of blood in the body, and the adaptability of vasomotor control.

Alternate exposures

It appears unlikely that the improved ability to withstand low environmental temperatures which was noted in the subjects of group I during the second period in the cold-room was due to a persistence of the effects of the first exposure, because the latter were abolished during the intervening period in the hot-room. Similarly, the improved adaptability to heat shown by the subjects of group II during the second period in the hot-room was probably not an after-effect of the first exposure to heat. It appears more likely that repeated changes of environmental temperature resulted in an improvement of the facility with which the volume and the distribution of the blood could be altered. Previous observations bear out this view. Thus the circulatory response of some men to exercise in a hot medium became more efficient after 1–2 weeks in a cool medium (Henschel, Taylor & Keys, 1943, Table 4), and superficial vasoconstriction in the extremities was found to set in more readily after repeated exposures to cold (Stein *et al.* 1949). Moreover, the Finnish *Sauna* (which consists of very hot and very cold baths), is considered to augment resistence to climatic extremes (Ott, 1948).

Practical conclusions

(1) An appreciable degree of acclimatization to cold can be achieved after 3 days in a moderately cool environment.

(2) Exercise or severe shivering during periods of cooling may inhibit acclimatization to cold.

(3) Frequent changes of temperature may be beneficial to those who must adapt themselves to extreme climatic conditions.

SUMMARY

1. Between the first and third day of exposure to cold there was a statistically significant increase of the skin temperature and the rectal temperature of 6 men. This was accompanied by greater subjective comfort, and it was taken to be a sign of acclimatization to cold.

2. Acclimatization to heat and cold was accompanied by apparent changes in the rate of superficial blood flow and the total blood volume, but it did not alter the distribution of blood in the body.

3. Repeated changes of the environmental temperature enhanced the facility with which the volume and the distribution of the blood could be varied.

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THE ISOLATION OF A HAEMOLYTIC SUBSTANCE FROM ANIMAL TISSUES AND ITS BIOLOGICAL PROPERTIES

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Attempts to explain the mechanism of erythrocyte destruction under normal and pathological conditions on immunological principles were not successful except in the case of paroxysmal haemoglobinuria (Donath & Landsteiner, 1904, 1905) and the anaemias involving the *Rh*-factor. Korschun & Morgenroth (1902) were the first to suggest that the destruction of erythrocytes might be due to the presence of a haemolytic substance in the blood affecting the erythrocytes directly without the interaction of an immunological factor, such as complement. They showed that a number of animal tissues contain an ether-soluble, heat-stable, non-antigenic fraction which is haemolytic *in vitro*. Their results have been confirmed and elaborated by Wölfel (1905), Levaditi (1903, 1905) and Friedemann (1909). Different results were reported by Belfanti (1924, 1925, 1928) who obtain an *ether-insoluble* haemolytic fraction from pancrease and, more recently, by Bergenhem & Fähraeus (1936), Bergenhem (1938), and Fähraeus (1939), who also obtained haemolytic activity in the ether-insoluble fraction of serum, the active principle of which they claimed to be either identical with, or closely related to, lysolecithin. The lecithinase responsible for its production in the body was supposed to manifest its activity in the spleen. Their theory has been accepted by a number of authors [Bogaert (1937), Gripwall (1938–9), Singer (1941), and Singer, Miller & Damaschek (1941)], especially in view of the clinical success of splenectomy in acholuric jaundice. On the other hand, Mann & Castle (1940), Foy & Kondi (1943–4), Gillespie (1944) and Maizels (1944–6) were either unable to confirm the lysolecithin nature of the haemolytic substance (H.S.) as described by Bergenhem & Fähraeus or rejected their evidence. It has, furthermore, to be realized that neither lysolecithin nor lecithinase has ever been isolated from plasma or tissue extracts. Lastly, the methods used for preparing the ether-insoluble H.S. are often open to criticism. Bogaert (1937), for instance, states that the lysolecithin-containing ether precipitate from serum was 'quickly dried at room temperature'

before using it for the haemolysis test. This procedure of drying either precipitates is unsatisfactory, as the resulting haemolysis from such preparations may easily be caused by the remaining traces of ether and not necessarily by the inherent haemolytic activity, a possibility which was not excluded by Bogaert.

In this paper the isolation of an ether-soluble H.S. (or a group of substances) from animal tissues is described, the biological properties of this substance are examined and its possible role in normal and pathological destruction of erythrocytes is discussed. Preliminary accounts of parts of this investigation have previously been given elsewhere (Laser & Friedmann, 1945; Laser, 1948).

METHODS

Preparation of erythrocyte suspension. Horse erythrocytes obtained from defibrinated blood of healthy animals, which were bled for serological work, have been used. A few ml. of erythrocytes were twice washed with saline or 0·15 M-phosphate buffer pH 7·3 and 5 of 10 % (v/v) suspensions prepared. A given suspension was divided into several parts and a fresh suspension used for each series to prevent protracted incubation at 37° before zero time. Erythrocytes in serum keep for 4–5 days in the cold, whereas washed erythrocytes are unreliable after the first day.

Suspension media and isotonic solutions of substances tested for their effect on haemolysis. The following molar concentrations were used according to the data given by Hitchcock & Dougan (1935) and Wilbur & Collier (1943): NaCl, 0·163 M; KCl, 0·15 M; $CaCl_2$, 0·11 M; $MgSO_4$, 0·155 M; NaF, 0·165 M; phosphate buffer (from Na_2HPO_4 and KH_2PO_4), 0·15 M; pyrophosphate, $Na_4P_2O_7$, 0·163 M in 0·163 N-HCl; metaphosphate, 0·15 M; sodium citrate, 0·109 M; sodium oxalate, 0·127 M; glucose, 0·296 M. The procedures adopted for preparing and testing certain organic substances were as follows. Crystalline albumin, globulin, haemoglobin and denatured globin were prepared according to the standard methods. Globin and stromatin were finely dispersed by vigorous shaking. Cholesterol (twice recrystallized) and lecithin (B.D.H.), dissolved in a small amount of ethanol, were added to the medium. The ethanol was then removed by boiling. Bilirubin, biliverdin, haematin, haematoporphyrin and protoporphyrin were dissolved in tribasic phosphate solution and neutralized with KH_2PO_4. They then remained in solution at neutral pH.

Preparation of stromata. These were obtained by high-speed centrifugation of laked (previously washed) erythrocytes or of washed erythrocytes laked with ice and ether (100 ml. erythrocytes + 30 ml. ice + 30 ml. ether + a few g. NaCl), whereby the stromata rise to the surface. They are then separated and, as far as possible, washed free from haemoglobin and dried *in vacuo*.

Measurement of haemolysis. All experiments, except those for the determination of the percentage haemolysis curve, were done in test-tubes at 37° in a water-bath having glass walls at the front and back. The test-tubes were placed in a metal stand which at its back held a transparent glass plate with horizontally etched and blackened lines at about 5 mm. distance apart. The test-tubes were viewed against a light source. End-points of haemolysis were reached when all cloudiness in the test-tube had disappeared and the lines on the glass plate became distinctly visible. Viewing the tubes through a red filter facilitated the determination of the end-point. The suspension medium, the H.S. in relatively high concentration, and the erythrocyte suspension were separately incubated in test-tubes until they attained the temperature of the bath. The haemolytic substance was then added to the test-tubes containing the medium, the erythrocytes being added last, at zero time. The contents of each test-tube were mixed by reversing the tube.

Qualitative test. A given amount of any fraction of the H.S. to be tested was taken up in a few ml. ether, or in 1 ml. ethanol, which was boiled down to about 0·2 ml. After addition of a known amount of water (if ether was being used the tubes were vigorously shaken) the tubes were immediately placed in boiling water for 5 min. The active material then formed a finely dispersed emulsion, a given amount of which was brought to isotonicity. Control tubes containing the same

amount of ether or ethanol were put up at the same time in order to check the complete removal of the solvent. This method was not applicable for quantitative determinations requiring known dilutions because after cooling some of the H.S. stuck to the walls of the test-tubes and to the pipettes, entailing an unknown loss.

Quantitative test. A known amount of the H.S. was dissolved with warming in 0·3M-phosphate (K_3PO_4 or Na_3PO_4) or in 0·32N-NaOH (or KOH). Stock solutions containing 0·5–1.0 mg./ml. were prepared. The stock solutions in alkali were stable in the cold, but on account of their marked thixotropy they had to be warmed before pipetting. To prevent a pH shift of the medium used for the measurement of haemolysis 0·163 ml. 0·15M-KH_2PO_4 or 0·1 ml. 0·32N-HCl were added per 0·1 ml. of the H.S. dissolved in K_3PO_4 or NaOH respectively.

Percentage haemolysis curves were determined in optical cuvettes at room temperature by means of a photoelectric cell measuring the transmitted light (King's colorimeter; red filter).

RESULTS

Attempts to demonstrate haemolytic activity in the ethanol-soluble and ether-insoluble fraction of serum, following the method of Bergenhem & Fähraeus (1936) failed, if care was taken to remove from the precipitate all traces of ether in a vacuum in the presence of shavings of paraffin wax. The ether precipitate from serum was, moreover, not found to be water soluble, as stated by Bergenhem & Fähraeus, and it was therefore tested in suspension. However, the ether-soluble fraction of serum possessed haemolytic activity and it was this fraction which was used for the isolation of the H.S.

Isolation of the haemolytic substance

Table 1 summarizes the general procedure of the isolation of H.S., which, in the first instance, was effected from serum. Later on, large-scale preparations were made from brain. The material obtained by high vacuum distillation, which is partly crystalline and partly oily at room temperature, is strongly haemolytic. This fraction has been used without further purification for the following activity tests and for examination of its biological properties.

TABLE 1. Isolation of the haemolytic fraction

(1) Dehydration of material with acetone or by freeze-drying
(2) Hot ethanol extraction of dried material
(3) Removal of ethanol. Dry residue obtained
(4) Ether extraction of residue suspended in water acidified with H_2SO_4 (blue to Congo red)
(5) Transference of alkali-soluble material from ether extract (4) into 1·5 % KOH solution
(6) Ether extraction of KOH fraction (5) acidified with H_2SO_4 (blue to Congo red)
(7) Transference of K_2CO_3-soluble material from ether extract (6) into 2·4 % K_2CO_3 solution
(8) Ether extraction of K_2CO_3-fraction (7) acidified with H_2SO_4 (blue to Congo red)
(9) Transference of alkali-soluble material from ether extract (8) into 1·5 % KOH solution
(10) Neutralization of KOH-fraction (9) with acetic acid and precipitation at neutral pH with 5 % lead acetate
(11) Drying of lead precipitate *in vacuo* and extraction with ether
(12) Decomposition of ether-soluble lead salts with dilute H_2SO_4
(13) Removal of ether. Dry residue obtained
(14) Molecular distillation of residue at high vacuum at as low a temperature as possible (about 60°)

A separate account of the further purification and chemical constitution of the H.S. will be given elsewhere in collaboration with Dr I. D. Morton, of the Chemical Laboratory, Cambridge. The purified substance, which was found

to contain only carbon, hydrogen and oxygen, is a monocarboxylic, mono-unsaturated fatty acid with the chain length C_{18}. (See note at the end of the paper.)

Distribution of haemolytic substance

H.S. has been found widely distributed in the body (Table 2). In every case purification has been carried through to the ether-soluble lead salt. High vacuum distillation has been carried out with the material from human plasma,

TABLE 2. Distribution of haemolytic substance in different tissues

Tissue	Relative content of H.S.
Plasma (human)	+
Serum (horse)	+
Spleen (horse)	+
Liver (horse)	+
Leucocytes (human pus)	+ +
Roe, male and female (herring)	±
Spermatozoa (hog)	−
Erythrocytes (horse)	+
Stromata	+
Remainder of cell	−
Erythrocytes (monkey) malaria parasitized*	+ + +
Brain—grey matter (human, horse)	+ + +
Brain—white matter (human, horse)	+ + +
Optic nerve (horse)	−
Anterior and posterior spinal roots (dog)	−

−, ±, +, + +, + + + =None, trace, small, medium and large yield.
* Laser (1948).

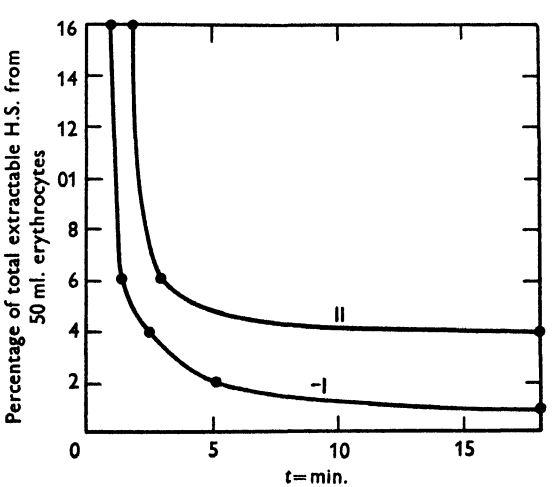

Fig. 1. Effect of incubation of erythrocytes (16 hr., 37°) on the yield of H.S. from stromata. In this and the following figures the abscissae give the time in minutes required for complete haemolysis. I, H.S. from normal erythrocytes; II, H.S. from incubated erythrocytes.

horse brain and malaria-infected monkey erythrocytes. While the content of H.S. in different tissues varied greatly, the haemolytic activity of the isolated material from different sources was of the same order. The data given in Table 2,

however, are only approximate, as they are based on comparatively rough overall calculations. Sperm (hog) was devoid of H.S. and herring roe contained only traces, which might be derived from the interstitial tissue. Leucocytes and brain (grey and white matter) gave the highest yield. The optic nerve (horse), however, and the anterior and posterior spinal roots (dog) had no recoverable H.S. In erythrocytes H.S. appeared to be bound to the stromata, the remainder of the cell having none. Incubation of erythrocytes at 37° prior to the separation of stromata decreased the amount of recoverable H.S. from the stromata (Fig. 1).

Aggregation of haemolytic substance at neutral pH

The activity of a given amount of H.S. was found to decrease with time after neutralization. This is probably the result of aggregation of H.S., as such preparations become slightly cloudy on standing. Deterioration of neutralized H.S. is more rapid the higher the concentration (Table 3). This fact has to be taken into account when evaluating the effect of inhibitors of H.S. In all subsequent experiments, therefore, the erythrocytes were added to each test-tube exactly 3 min. after the H.S.

TABLE 3. Deterioration of activity of haemolytic substance after neutralization

Time between addition of erythrocytes and neutralization (min.)	Vol. in which neutralized (ml.)	Haemolysis time (min.)
0	10	2·25
15	10	3·5
15	0·3	6·0
150	0·3	23·0

H.S., 1:200,000, final concentration in 10 ml. 0·1 ml. 7·5 % erythrocyte suspension.

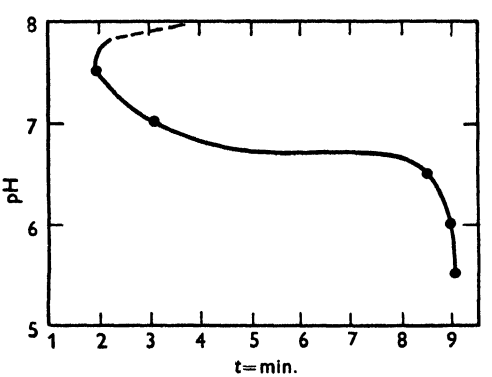

Fig. 2. Effect of pH on the rate of haemolysis by H.S. H.S., 1:120,000 in 10 ml. 0·15 M-phosphate buffer; 0·1 ml. 7·5 % erythrocyte suspension.

Effect of pH

Fig. 2 shows the dependence of the activity of H.S. on the pH of the medium. In view of the aggregation, with consequent deterioration of the activity of

H.S. at and below neutral pH, it cannot be decided whether the variations of haemolysis time with pH are real or apparent, because it is possible that aggregation of H.S. occurs faster at lower pH. However, no further delay of haemolysis time occurs between pH 6 and 5, furthermore, towards alkaline pH haemolysis again tends to slow down. At still higher pH values lysis takes place in the absence of H.S. Regardless of interpretation, however, the data prove the necessity of controlling the pH in *in vitro* experiments with haemolytic substances.

Effect of phosphate concentration

The rate of haemolysis increases with increasing phosphate concentration in saline in a manner shown in Fig. 3. The effect is equally obtained if orthophosphate is replaced by either pyrophosphate, metaphosphate or adenosine-

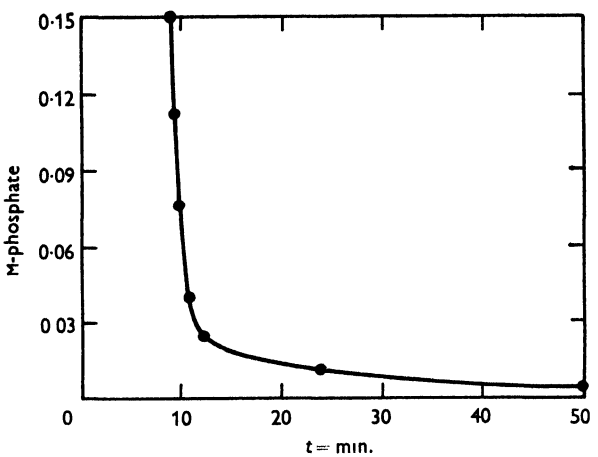

Fig. 3. Effect of phosphate concentration on the rate of haemolysis by H.S. in an isotonic medium containing phosphate buffer and sodium chloride. H.S., 1:200,000 in 10 ml. isotonic medium; 0·1 ml. 10 % erythrocyte suspension.

triphosphate. A certain concentration of phosphate does seem to be necessary for optimal activity of H.S., although no definite ratio of phosphate to the amount of H.S. or of erythrocytes has been established. The phosphate effect can be demonstrated with regularity only with low concentrations of H.S. acting on fresh erythrocytes. A similar phosphate effect has been obtained with saponin as the haemolytic agent (Fig. 4), a result which is also evident in the experiments of Maizels (1944–6).

Effect of other substances

The substances examined are listed in Table 4. Since H.S. used for this series had been dissolved in K_3PO_4, all tubes contained a small amount of phosphate buffer (final concentration 0·004 M, pH 7·3), which by itself, however, is too small to produce the phosphate effect. The tested substances can be classified

in three groups: (*a*) those which do not affect the rate of haemolysis compared with that in saline: these are KCl and glucose; (*b*) those which slow down the rate of haemolysis: these are $CaCl_2$ and $MgSO_4$; (*c*) those which accelerate

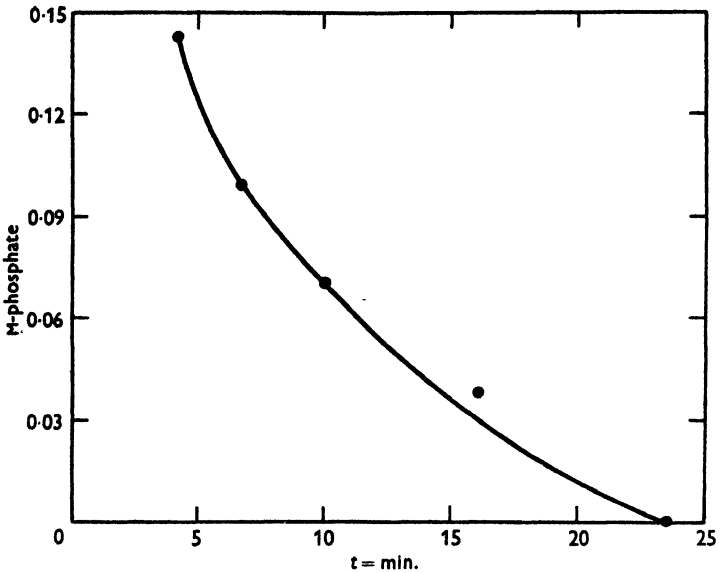

Fig. 4. Effect of phosphate concentration on the rate of haemolysis by saponin in an isotonic medium containing phosphate buffer and sodium chloride. Saponin, 1:125,000 in 10 ml. isotonic medium; 0·2 ml. 10 % erythrocyte suspension.

haemolysis to about the same extent as phosphate in optimal concentration: these are Na-citrate and oxalate. The acceleration caused by Na-fluoride was less pronounced.

TABLE 4. Effect of various substances on haemolysis time

Substance tested	Haemolysis time (min.)
—	29
Phosphate	8
KCl	33
Glucose	38
$CaCl_2$	~
$MgSO_4$	90
Na-citrate	7
Na-oxalate	5
Na-fluoride	15

H.S. 1/200,000 final concentration. 0·2 ml. 5 % erythrocyte suspension. The samples contained 0·004 M-phosphate buffer pH 7·3 (for dissolving and neutralizing H.S.), and 1·2 ml. isotonic solution of the substance to be tested in 10 ml. isotonic saline.

The inhibitory effect of Ca and Mg arises probably from the formation of insoluble Ca or Mg salts of H.S., while the speeding up caused by citrate, oxalate and fluoride is probably the result of the combination of these substances with

Ca on the cell surface, and its removal as a natural brake on the rate of haemolysis. A similar acceleration of saponin haemolysis by Na-oxalate and citrate has also been observed by Wilbur & Collier (1943), who explain the effect as due to initial shrinkage of the erythrocytes in Na-oxalate and citrate solutions, a condition which accelerates haemolysis. Their measurements were done in isotonic Na-oxalate or citrate solutions; in the experiments just described isotonic Na-oxalate and citrate solutions had been diluted eight times. It seems therefore desirable, when studying haemolysis, to refrain from using anticoagulants such as sodium citrate or oxalate, but to use either defibrinated blood or heparin, which does not affect the haemolysis rate.

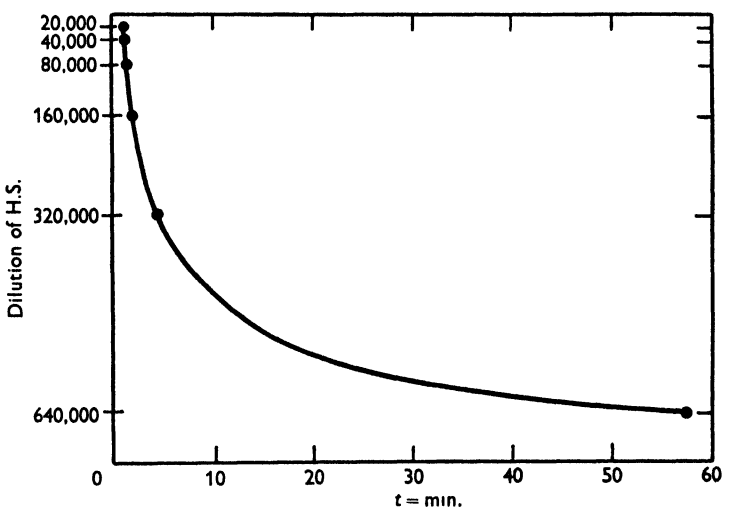

Fig. 5. Relationship between dilution of H.S. and rate of haemolysis. 10 ml. phosphate buffer pH 7·3; 0·1 ml. 5 % erythrocyte suspension.

Relationship between dilution and haemolysis time

Time-dilution curves have been obtained by varying either the amount of erythrocytes acted upon by the same amount of H.S. or by varying the amount of H.S. acting on the same amount of erythrocytes (Fig. 5). The curves follow the same pattern as described by Ponder (1934) in experiments with saponin.

It has been observed by several workers that a given amount of a haemolytic substance which haemolyses x ml. erythrocytes in y min. haemolyses consecutively added fractions of x ml. erythrocytes in the aggregate in a longer time (Ponder, 1932). The haemolysis by H.S. behaves in a similar way (Fig. 6). It is assumed that some products set free by haemolysis, such as stromata and haemoglobin act as inhibitors. This is borne out by a test, in which the haemolysis time was measured in presence of a laked 10 % erythrocyte suspension with and without the removal of the stromata. The haemolysis time of the

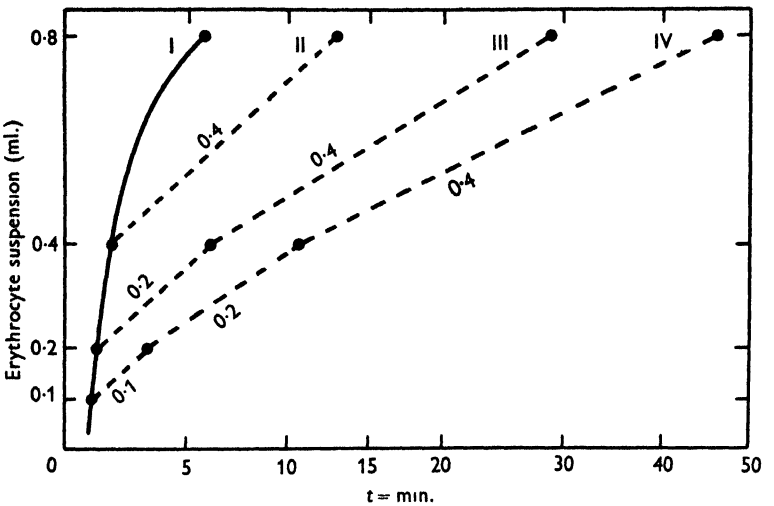

Fig. 6. Relationship between constant amount of H.S. and varying amounts of erythrocytes, when added in fractions. H.S., 1:100,000 in 10 ml.; phosphate buffer pH 7·3; 5 % erythrocyte suspension. Dotted lines indicate consecutive addition of erythrocyte suspension, each following addition being made immediately haemolysis of the preceding amount of erythrocytes has been completed. I, addition to four different tubes of 0·1, 0·2, 0·4 and 0·8 ml. erythrocyte suspension (solid line); II, consecutive addition to the same tube of 0·4 and 0·4 ml. erythrocyte suspension; III, as II with 0·2, 0·2 and 0·4 ml. erythrocyte suspension; IV, as II with 0·1, 0·1, 0·2 and 0·4 ml. erythrocyte suspension.

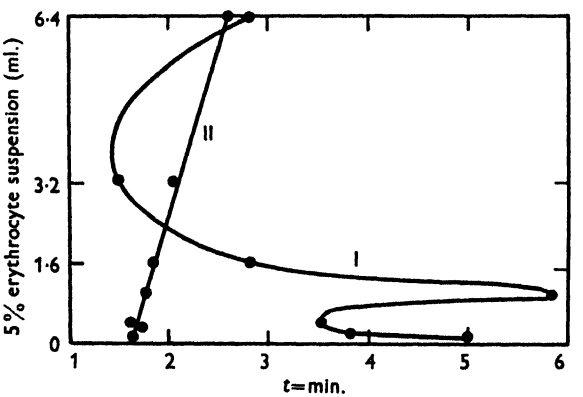

Fig. 7. Effect of the addition of laked erythrocytes on the time of haemolysis with increasing amounts of erythrocytes and constant amounts of lysolecithin. Lysolecithin, 1:4000 in 10 ml. phosphate buffer pH 7·3; 5 % erythrocyte suspension.

Tubes ...	1	2	3	4	5	6	7
Series I: erythrocyte suspension (ml.)	0·1	0·2	0·4	0 8	1·6	3·2	6·4
Series II: erythrocyte suspension (ml.) + laked	0·1	0·2	0·4	0·8	1·6	3·2	6·4
erythrocytes (ml.) (5 % suspension)	6·3	6·2	6·0	5·6	4·8	3·2	0

control (0·1 ml. 10 % erythrocyte suspension in 10 ml. phosphate pH 7·3, H.S. 1:100,000) was 1·7 min.; in presence of 0·4 ml. laked erythrocytes it was 16 min.; while in presence of 0·4 laked erythrocytes with stromata removed it was 4 min. Hence both the stromata and some constituents of the erythrocytes decreased the rate of haemolysis.

It is interesting to note that the time-dilution curve with lysolecithin differs fundamentally from that obtained with H.S. The rate of haemolysis shows an optimum with low concentrations of lysolecithin relative to the amount of erythrocytes, i.e. a given amount of lysolecithin lyses a large amount of erythrocytes more quickly than a small amount (Fig. 7, curve I). It thus seems that the products of haemolysis accelerate erythrocyte destruction by lysolecithin, contrary to their effect on H.S. haemolysis. This is borne out by the experiment shown in Fig. 7, curve II, where the addition of haemolysed erythrocytes increases the lysolecithin haemolysis rate. The mechanism of lysolecithin haemolysis is therefore different from that operating with H.S.

The percentage haemolysis curve

A calibration curve was first established by measuring the light transmitted when using erythrocyte suspensions and haemolysed erythrocytes in varying proportions. Details of the procedure for making up standards of a given

TABLE 5. Procedure for obtaining erythrocyte suspensions representing various percentage of haemolysis for determining the percentage haemolysis curve (calibration curve) with the King colorimeter

Sample	0·5 % erythrocyte in phosphate buffer (ml.) (1)	Water (ml.) (2)	Twice isotonic phosphate buffer buffer (ml.) (3)	Isotonic saline (ml.) (4)
I	3·0	—	—	12·0
II	2·5	7·5	7·5	2·5
III	5·0	10·0	10·0	5·0
IV	7·5	12·5	12·5	7·5
V	3·0	12·0	—	—

Sample	0·5 % erythrocyte in phosphate buffer (ml.) (5)	Isotonic saline (ml.) (6)	Cuvette mixture from (1)+(2)+(3)+(4) (ml.) (7*a*)	Cuvette mixture from (5)+(6) (ml.) (7*b*)	Representing percentage haemolysis of 0·1 % erythrocyte suspension (8)
I	—	—	5·0	—	0
II	5·0	15·0	2·0	3·0	25
III	4·0	12·0	3·0	2·0	50
IV	2·5	7·5	4·0	1·0	75
V	—	—	5·0	—	100

Five samples (I–V) representing the required percentage of haemolysis (column (8)) were obtained by the addition of varying amounts of erythrocyte suspensions to isotonic solutions of haemolysed erythrocytes of varying concentration. This was effected by separately mixing for each sample (I–V) the volumes stated in columns (1)+(2)+(3)+(4) and (5)+(6). The optical cuvettes, which held 5 ml. were then filled with varying amounts of these mixtures, as indicated in column (7*a*) and (7*b*).

erythrocyte suspension which contain known percentages, say 50 % of intact and 50 % of haemolysed cells in isotonic solution, are given in Table 5. The calibration curve thus obtained was found to be a straight line. H.S. was added to an erythrocyte suspension of the same concentration and galvanometers readings taken at convenient intervals, depending on the speed of the reaction. The readings were then converted into percentage haemolysis on the basis of the calibration curve. Fig. 8 shows the results obtained with different amounts of H.S.

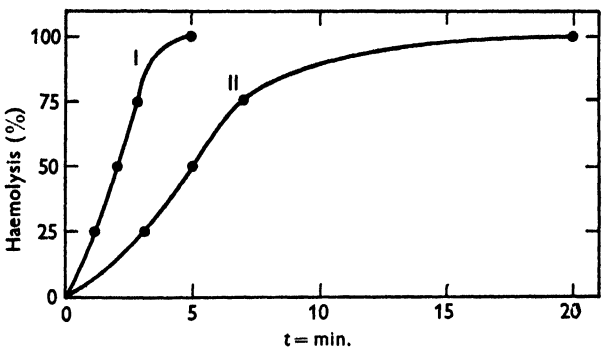

Fig. 8. Percentage haemolysis curves, determined photometrically with the King colorimeter. $T = 17°$. Empirical amounts of H.S.: concentration in test I about three times that in test II. 5 ml. 0·1 % erythrocyte suspension in phosphate buffer pH 7·3.

Inhibitors of haemolytic substance present in plasma

Normal plasma contains H.S., and relatively large amounts of H.S. can be injected intravenously without any apparent haemolysis. It is therefore likely that plasma contains inhibitors of H.S. The following physiological components of plasma have been examined: albumin, globulin, bilirubin and cholesterol. The proteins and cholesterol proved to be strong inhibitors, bilirubin had a slight inhibitory effect in fairly high concentration (> 1/10,000). Globulin inhibits less than albumin (Fig. 9). The strong inhibition by calcium has already been mentioned. In the case of cholesterol it is difficult to decide whether the inhibitory effect is specific or a result of the physical properties of the suspension of cholesterol which adsorbs H.S. in an unspecific way. However, even adsorbed H.S. should come into contact with erythrocytes. As at a concentration of cholesterol of 1/100,000 inhibition was infinite, there is no reason to doubt its specific inhibitory effect, which has also been observed in saponin haemolysis by Ranson (1901) and Stocks (1919–20). The inhibitory effect of serum on the H.S. haemolysis, determined quantitatively by serial dilutions *in vitro*, was found to correspond closely to the effect produced by the equivalent amount of albumin contained in the serum. The inhibition of H.S. by plasma *in vivo* seems, therefore, to be accounted for by its protein content and not by cholesterol.

Inhibitors of haemolytic substance present within the erythrocytes

Since H.S. occurs in normal erythrocytes and can be extracted from their stromata after washing and laking it must be assumed that H.S. is inactive while bound to the stromata. In order to test the possibility that other constituents of the erythrocytes might act as inhibitors of H.S., the following substances were examined: haemoglobin, lecithin and biliverdin, the last-named being the substance most closely related to verdohaematin, the first normal breakdown product of haemoglobin (Lemberg, Cortis-Jones & Norrie, 1938*a*, *b*). Biliverdin did not affect the rate of haemolysis by H.S., while

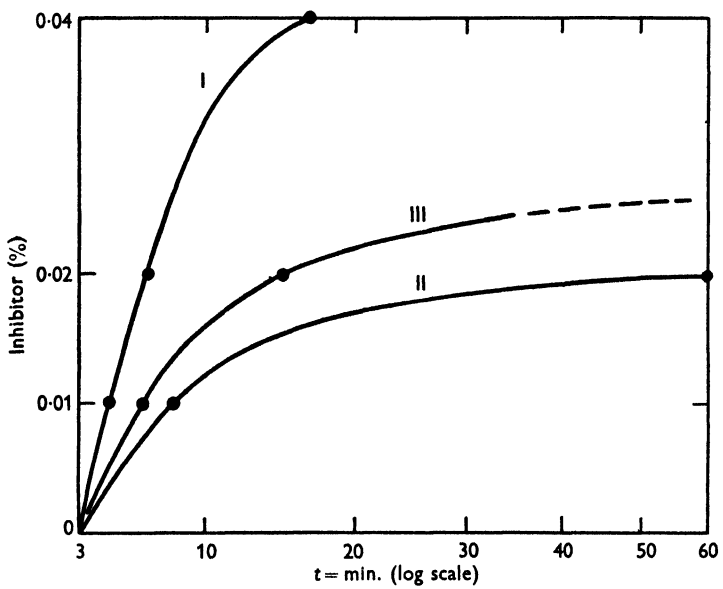

Fig. 9. Relationship between concentration of inhibitor and time of H.S. haemolysis. H.S., 1/50,000 in 10 ml. phosphate buffer pH 7·3; 0·5 ml. 5 % erythrocyte suspension. I, globulin; II, albumin; III, serum, calculated as protein equivalent.

haemoglobin and lecithin were found to act as inhibitors. Under the experimental conditions (H.S. 1/200,000) lecithin inhibited only at a concentration > 1/100,000, while haemoglobin was effective at a concentration > 1/100,000. Assuming a molecular weight of H.S. of about 300, the inhibitory effect of lecithin seems to occur at roughly equimolar concentration of lecithin and H.S., while the inhibition by haemoglobin was manifest at a much lower molar concentration. The inhibition by lecithin was not apparently caused by impurities in the lecithin preparation, as these would mainly be free fatty acids derived from lecithin which are known to have the reverse effect. In fact, several samples of old or less pure lecithin either increased the rate of haemolysis by H.S. or were haemolytic in absence of H.S.

Haematin and porphyrins

Haematin, which is not a physiological breakdown product of haemoglobin, was found by itself to be haemolytic *in vitro* down to relatively low concentra-

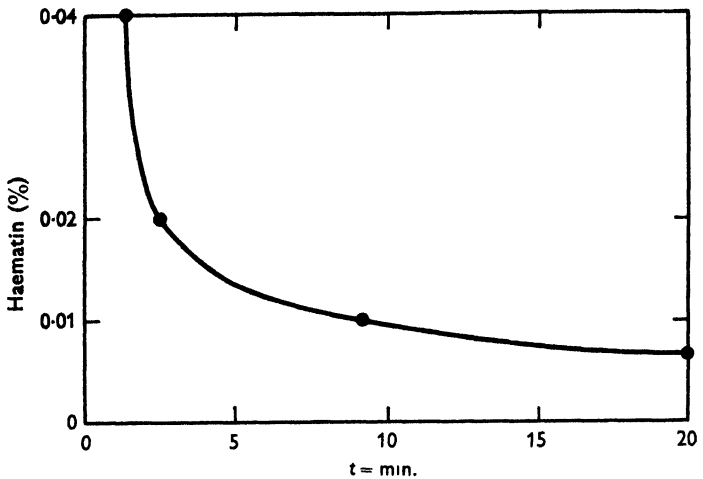

Fig. 10. Haemolytic effect of haematin. Relationship between concentration of haematin and haemolysis time. 0·1 ml. 5 % erythrocyte suspension in 10 ml. phosphate buffer pH 7·3.

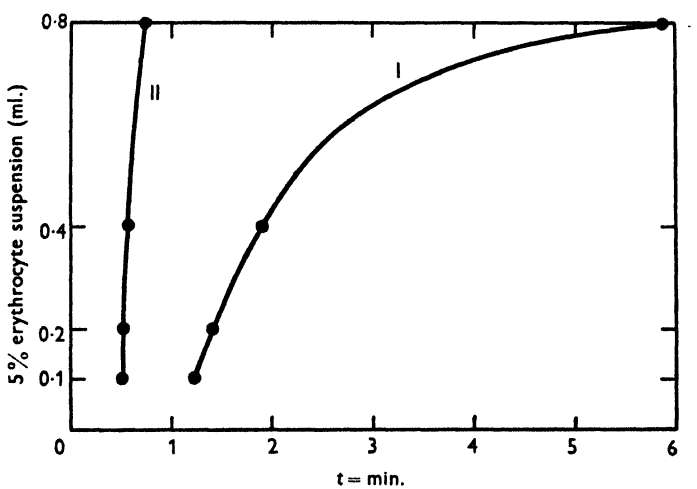

Fig. 11. Effect of subhaemolytic concentration of haematin on H.S. haemolysis. H.S. 1/100,000 in 10 ml. phosphate buffer pH 7·3; 5 % erythrocyte suspension. I, time of H.S. haemolysis with increasing amounts of erythrocytes; II, as I in the presence of haematin 1/100,000.

tions (Fig. 10). In subhaemolytic concentrations it considerably increased the rate of haemolysis by H.S. (Fig. 11). This effect was enhanced in the presence of lecithin (Table 6) although lecithin itself acted as an inhibitor. In high

concentration haematin abolished the inhibition of H.S. haemolysis by serum (Table 7). Cyan-haematin and glyoxaline-haematin act like haematin, while haemato-porphyrin and protoporphyrin had a similar but less pronounced effect requiring higher concentrations. The effect of haematin and its power to increase H.S. haemolysis were dependent upon the presence of phosphate.

TABLE 6. Effect of haematin in subhaemolytic concentration of haemolytic substance haemolysis in presence of added lecithin

	Haemolysis time (min.)
H.S.	13
H.S. + haematin	2
H.S. + lecithin	>90
H.S. + haematin + lecithin	0·66

H.S., 1/200,000 in 10 m. phosphate buffer pH 7·3. Lecithin, 1/40,000. Haematin, 1/100,000. 0·125 ml. 10 % erythrocyte suspension.

TABLE 7. Effect of haematin on inhibition of haemolytic substance haemolysis by serum

	Haemolysis time (min.)
H.S.	3
H.S. + serum (200)	~
H.S. + haematin (2,500)	0·5
H.S. + haematin (5,000)	0·75
H.S. + haematin (10,000)	1·0
H.S. + serum (200) + haematin (2,500)	0·5
H.S. + serum (200) + haematin (5,000)	1·3
H.S. + serum (200) + haematin (10,000)	7·5

H.S., 1:100,000 in 10 ml. phosphate buffer pH 7·3. 0·4 ml. 5 % erythrocyte suspension. Figures in brackets denote dilutions.

Phenylhydrazine

Phenylhydrazine is known to produce an increase in the number of siderocytes. These are aged erythrocytes which are easily disposed of in the body by phagocytosis. Phenylhydrazine, therefore, accelerates ageing and increases the osmotic fragility of erythrocytes. In high dilutions (1/100,000–1/200,000) it was found to accelerate the rate of H.S. haemolysis by about 50 %. No further acceleration could be produced by increasing the concentration or by previous incubation of erythrocytes with phenylhydrazine. The effect of phenylhydrazine was furthermore found to be irreversible.

DISCUSSION

At the time when Korschun & Morgenroth (1902) described the existence in extracts of animal tissues of an ether-soluble haemolytic substance, which was not antigenic and acted without complement, the main interest connected with problems of haemolysis lay in the field of immunology. Their work has therefore not been followed up. After the elucidation of the haemolytic effect of cobra venom, which causes lysolecithin to be formed by means of a lecithinase

(Delezenne & Fourneau, 1914), attempts were made to demonstrate the existence of lysolecithin in certain tissue extracts (Belfanti, 1924) and in incubated serum (Bergenhem & Fähraeus, 1936). These claims, however, have not been supported by more recent investigations. For instance, Gillespie (1944) found that the addition of lysolecithin to suspensions of erythrocytes in serum produced neither an increase in volume nor in osmotic fragility of erythrocytes, effects which are the characteristic of incubation with serum. There is, in fact, no convincing evidence that it is possible to extract from tissues or from normal or incubated serum a haemolytic substance of the nature of lysolecithin. In the present experiment it has also not been possible to obtain an ether-insoluble haemolytic fraction showing the chemical or biological properties of lysolecithin.

The haemolytic substance described in this paper was first isolated from the ethanol—and ether-soluble fraction of plasma (Laser & Friedmann, 1945). It has also been found to occur in other tissues, the largest yield being obtained from brain.

The chemical constitution of the substance has not yet been established beyond the fact that it is a mono-unsaturated, mono-carboxylic fatty acid with a chain length C_{18}. (See note at the end of the paper.) Fatty acids are known to cause haemolysis *in vivo*, as in *Bothriocephalus* anaemia (Faust & Tallquist, 1907), and also to be haemolytic *in vitro*. However, the minimal haemolytic concentration of oleic acid and of the acid isolated in these investigations are of a different order, the latter substance being considerably more powerful.

The optimal activity of H.S. *in vitro* was found to be dependent on the presence of phosphate. This effect might be regarded as similar to that of citrate or oxalate, i.e. removal of the inhibition by Ca through formation of an insoluble Ca salt. Analysis of the conflicting claims for the absence or presence of Ca in erythrocytes seems to justify the conclusion that they contain Ca in very much lower concentration than the plasma. A similar suggestion of the importance of intracellular phosphate for saponin haemolysis has already been put forward by Port (1910), Höber & Nast (1914), and Orahovats (1926). Ponder (1927), however, has rejected the possibility of a relation between P content and haemolysis rate as, according to his mathematical treatment of haemolysis as a simple reaction, the amount of any substance which is involved in a haemolytic process should not be directly but inversely proportional to its degree of lability. Yet the phosphate effect could clearly be demonstrated with H.S. as well as with saponin. There appears to be no reason to doubt that an increased intracellular content of P should similarly facilitate haemolysis. The objection of Ponder may therefore not hold, since a system, which involves accelerators of haemolysis, is a complex one, to which the mathematical treatment of a simple haemolytic reaction does not apply.

At present it is difficult to make any assumptions regarding the physiological significance of the presence of H.S. in relatively large amounts in different organs, especially brain, or regarding its distribution in the central nervous system. However, the existence of H.S. inside the erythrocyte lends support to the assumption that H.S. is concerned in the normal destruction of erythrocytes. Owing to the large excess of inhibitors of H.S. in the plasma, notably albumin, it cannot be assumed that any circulating H.S. is concerned in normal erythrocyte destruction, which takes place as an inherent function of the erythrocytes, as evidenced by the linear destruction of donor erythrocytes when transfused into normal patients or into patients suffering from anaemias, including pernicious anaemia and congenital familial acholuric jaundice (Brown, Hayward, Powell & Witts, 1944; Callender, Powell & Witts, 1945) or the linear decay of sulphaemoglobin erythrocytes in T.N.T. workers (Jope, 1946). However, in the reactions which determine the life span of the erythrocyte H.S. is probably an important factor. The yield of H.S. recoverable from stromata decreases on incubation of the erythrocytes in serum. This may be explained on the assumption that bound H.S. is liberated on autolysis from the stromata into the corpuscle. In a similar manner bound inactive H.S. might be liberated *in vivo* into the ageing corpuscle. If this were to happen at or near the surface of the erythrocyte, its increased fragility or haemolytic destruction might easily result.

None of the normal constituents of plasma or erythrocytes so far tested accelerated H.S. haemolysis, some were ineffective, while most of them were inhibitory, especially haemoglobin and albumin. A qualitatively different result was obtained with haematin, haematoporphyrin and protoporphyrin which do not occur under physiological conditions in the body. In certain concentrations they proved to be haemolytic by themselves and in subhaemolytic concentration to accentuate the effect of H.S. The acceleration of H.S. haemolysis by haematin in subhaemolytic concentration may be specific for H.S. and substances of similar constitution since it was shown that under similar conditions saponin haemolysis was delayed.

Haematin has been found in the plasma in a number of cases of pernicious and haemolytic anaemias (Heilmeyer, 1932, 1943) and porphyrin has been isolated in a case of haemolytic anaemia by Fischer & Zerweck (1924*a*, *b*) and by Müller (1931). According to Heilmeyer the occurrence of haematin is not due to excessive destruction of erythrocytes but to a particular disturbance of the normal degradation process of haemoglobin, leading to abnormal products. The formation of haematin in these cases is generally believed to occur in the plasma from liberated haemoglobin. The evidence brought forward in the present paper that haematin is haemolytic by itself, that in subhaemolytic concentrations it increases the haemolytic power of H.S., and that this effect is strongly potentiated by the presence of lecithin, may have a certain bearing

on those pathological conditions of increased erythrocyte destruction which are associated with the appearance of haematin and, possibly, some porphyrins. It is suggestive to assume that in these cases the occurrence of haematin might not only be the result of erythrocyte destruction but one of its causes.

SUMMARY

1. A haemolytic substance of high activity has been isolated from plasma and a number of animal organs.

2. Chemically the substance appears to be a mono-unsaturated, mono-carboxylic fatty acid with the chain length C_{18}.*

3. The haemolytic substance is widely distributed in the body. The relative content varies considerably in different organs and in different parts of the same organ, as, for example, in the central nervous system. In erythrocytes the substance is bound to, and inactivated by, the stromata.

4. Optimal haemolytic activity of the substance *in vitro* is dependent upon the presence of phosphate.

5. The body contains a large number of inhibitors of the haemolytic activity of the substance, notably proteins, cholesterol, lecithin and calcium, while none of a large number of normal constituents of plasma or erythrocytes accentuates its effect.

6. Haematin was found to be haemolytic by itself and in subhaemolytic concentration considerably to increase the effect of the haemolytic substance, especially in the presence of lecithin.

7. The presence of the haemolytic substance inside the erythrocytes is assumed to be related to their normal life span.

8. The existence of lysolecithin in the body, which has been claimed, has not been confirmed.

* Note added on 26 August 1949. The acid has been identified by Dr I. D. Morton as *cis*-11–12 octadecenoic acid [$CH_3.(CH_2)_5CH=CH.(CH_2)_9.COOH$] and this has been confirmed by synthesis.

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BASAL METABOLIC RATES AND PHYSICAL FITNESS SCORES OF BRITISH AND INDIAN MALES IN THE TROPICS

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Since the early and apparently contradictory observations of Eijkmann (1896) and Almeida (1920) on the basal metabolic rate (B.M.R.) of people living in the tropics, various other investigations have been carried out, principally to determine whether there is a racial factor in metabolism, but also to assess how far the environment has an effect. The literature on the B.M.R. of different races in various environments has been reviewed by DuBois (1936) and more recently by Wilson (1945). Although the evidence suggests that there is, in the tropics, a deviation below normal North American standards, the results obtained are by no means consistent.

Varying results have also been obtained when the values of other physiological functions in the tropics and in the temperate zone have been compared. Roddis & Cooper (1926), Knipping (1923) and Sayers & Harrington (1923) found that the systolic blood pressure of people accustomed to living in the temperate zone tended to fall during a stay in the tropics. On the other hand, MacGregor & Loh (1941) found little difference in British soldiers stationed for 6 months and for 2½ years respectively in the tropics.

No comparison appears to have been made so far between the physical efficiencies of similar groups of people during increasing sojourn in the tropics, and only a few observations have been made on the fitness for muscular work of natives and of white settlers living under similar tropical conditions. Cluver, de Jongh & Jokl (1942) observed that Bantu children made higher scores on physical fitness tests than did white children from the same locality, even although the Bantus were living on a lower economic level and their diet was often inadequate.

In view of the inconsistence, and in some respects absence, of data concerning the effects of tropical residence on human physiological characteristics, the

opportunity was taken whilst in India to make some measurements on Indian and European personnel of the Royal Air Force, living at base under very similar service conditions. The latter did not give the complete identity of environment desirable for a true racial comparison, but they provided reasonable similarity for some months in respect of food, exercise, sleep and hygiene. Measurements were also made on other subjects from the tropics, after continued exposure to cold climatic conditions.

METHODS

Measurements were made of basal metabolic rate, blood pressure, pulse rate, body temperature and physical fitness.

Basal metabolic rate. The 'Field Respiration Apparatus' devised by Benedict was used for measuring the basal metabolism. This apparatus, which is portable and of the closed circuit type, was designed primarily for surveys of racial metabolism (Benedict, 1927). The basal metabolic rate, in every case, was calculated from the first determination of the oxygen consumption. In assessing the degree of deviation from the normal in temperate regions, reference was made to the Aub-DuBois (1917) and to the Boothby, Berkson & Dunn (1936) standards. Both standards are derived from measurements on normal subjects in North America.

Physical fitness tests. The Harvard Pack test (Johnson, Brouha & Darling, 1942) was mainly used to provide an index of physical fitness, this being calculated as follows:

$$\text{Fitness index} = \frac{\text{duration of the exercise in seconds} \times 100}{\text{twice the sum of the pulse counts in intervals } 1\text{–}1\tfrac{1}{2},\ 2\text{–}2\tfrac{1}{2},\ 4\text{–}4\tfrac{1}{2} \text{ min. after excercise}}.$$

Johnson and his colleagues have graded physical condition in relation to this index thus:

	Physical condition
Below 50	Poor
50–75	Fair
Above 75	Average
75–90	Good
Above 90	Superior

This test is one of fitness for hard physical work. The physical fitness ratio test which is used in the Royal Air Force for assessing fitness gives weight to both agility and strength. The score is assessed from (*a*) the number of pull-ups and sit-ups which can be carried out, and (*b*) the time required to run a certain distance. Details of this test are given on the R.A.F. Physical Fitness Test Record Card (Form 1835) which may be obtained from the Inspectorate of Physical Fitness, Adastral House, Kingsway, London. Only the British physical training instructors performed this test.

Procedure. At Bombay sleeping quarters were provided for the subjects and the physiological measurements were made in an adjacent room. On other stations the men slept in their own quarters but all measurements, other than those of physical fitness, were made on men assembled before breakfast. When basal conditions were required, the last meal was taken not less than 10 hr. before the test. Those men performing the fitness tests, however, always had a meal between 1 and 2 hr. previously. They performed the pack test indoors and the physical fitness ratio test outdoors.

The room used for the measurements contained two Indian string beds with the metabolism apparatus and the other measuring instruments conveniently between them. One subject could thus see what was happening to the other in the course of a determination, which helped to allay possible nervousness. The estimation of the basal metabolism was begun after the man had been lying down for 30 min. and it was completed approximately 15 min. later. The pulse rate was then taken and immediately afterwards systolic and diastolic blood pressures were measured using the

auscultatory method. Finally a thermometer was introduced into the rectum, left for 5 min., and a reading taken.

In order to provide a reasonably warm environment for the tests in Kashmir, a room was heated as well as possible, namely to 55° F. This air temperature is considerably lower than that prevailing in the tropics (75–85° F.) and the tests in the two environments are not from this point of view strictly comparable. However, any effect of this particular environmental difference was minimized by the fact that the subjects were very warmly clad and in addition were covered with a blanket whilst the measurements were in progress. Also radiant heat was supplied by the stove.

Subjects. The basal metabolic rate and certain other measurements in the basal state of eighty-five British airmen in Bombay were obtained. Similar measurements were made on forty Indian airmen, who in addition performed the Harvard Pack test. This group included twenty-one physical training instructors and nineteen clerks. Half of each occupational category came from the Punjab and the United Provinces and the other half from Bombay and Madras.

Measurements were also made on twenty British aircrew personnel from tropical India staying for a month under cold climatic conditions at a mountain skiing centre in Kashmir. For comparison three British skiing instructors who had been in the area for about 6 months and three Kashmiri hill porters were tested.

The following groups performed the Harvard Pack test only:

(*a*) Thirty British aircrew personnel who had been in India for about 3 months, and twenty-one Indian aircrew personnel.

(*b*) Twenty British clerks who had been in India for periods between 6 months and 3 years.

(*c*) Fifty-three British physical training instructors with tropical service varying from 6 months to 4 years. This group also performed the physical fitness ratio test.

TABLE 1. Comparison of physical and physiological characteristics of British and Indian airmen in the tropics. Group means and standard errors are given

	Race	
	British	Indian
Number of subjects ...	78	40
Age (years)	24·7±0·5	21·6±0·5
Weight (lb.)	140·1±1·6	125·0±2·0
Height (in.)	68·7±0·3	67·0±0·4
Ratio of Weight to Height	2·04	1·88
Basal metabolism:		
Oxygen consumption (c.c./min.)	231·6±2·8	209·0±3·9
Kg.cal./sq.m./hr.	37·4±0·4	36·3±0·6
Percentage of Aub-DuBois standard	94·4±1·1	90·4±1·3
Percentage of Boothby-Berkson-Dunn standard	92·8±1·1	88·6±1·3
Blood pressure:		
Systolic pressure (mm. Hg)	114·3±1·3	103·0±1·7
Diastolic pressure (mm. Hg)	74±1·0	65·3±1·1
Pulse pressure (mm. Hg)	40·3	37·7
Pulse:		
Frequency/min.	64·0±1·0	67·0±1·6
Temperature (° F.):		
Rectal	98·30±0·05	—
Mouth	97·50±0·06	97·95±0·08

RESULTS

British subjects in tropical India

The Bombay group had been in the tropics for an average time of 10·7 months, their individual lengths of service ranging from 6 weeks to 3 years. Table 1 shows the mean values of the measurements obtained for this group and also for the group of forty Indian airmen. The B.M.R. of the British group is 5·6%

below the Aub-DuBois standard and 7·2% below the Boothby-Berkson-Dunn standard. The mean pulse rate is slightly higher than that of subjects living in a temperate climate (Benedict, 1928). The systolic blood pressure of the group is lower than the figure of 126·5 mm. obtained by Alvarez (1920) from measurements in the U.S.A. on 2930 men between the ages of 16 and 40, but these subjects were not under basal conditions at the time of measurement. Although the environmental conditions were exacting—air temperatures between 83 and 88° F. and relative humidities up to 80%—in only ten instances did the rectal temperature reach 99° F. and in only one instance was it as high as 99·2° F. The mean oral temperature of the subjects was 0·8° F. less than the mean rectal temperature.

TABLE 2. Weight, height and age of groups of British airmen with different lengths of tropical service. Group means and standard errors are given

Months in tropics ...	1–3	3–12	12–24	24–36
Number in groups	18	41	15	11
Percentage of total subjects in each group	21·2	48·2	17·6	12·9
Mean weight (lb.)	144·9±2·5	137·6±2·4	139·2±2·0	142·8±5·4
Mean height (in.)	69·5±0·5	68·0±0·3	68·4±0·6	70·1±1·2
Mean age	23·7±0·7	23·8±0·6	24·8±1·4	29·1±1·9

When individual deviations from the Aub-DuBois standard values were plotted against the corresponding lengths of stay in the tropics, the relation shown in Fig. 1 was obtained. Four different lengths of tropical residence were considered (Table 2). The mean B.M.R. for each is shown in Table 3. Fig. 2*a*, *b* give the data in histogram form for comparison with other values for British and Indians. Calculation of the correlation coefficient using the values derived from the Aub-DuBois standard for B.M.R. gave a value, $r = -0{\cdot}25 \pm 0{\cdot}11$, which is significant ($P < 0{\cdot}05$), the regression equation for B.M.R. on time in years being $y = 97{\cdot}21 - 2{\cdot}93x$. But, using the same data for heat output, expressed as a deviation from the more recently established Boothby-Berkson-Dunn standard, the correlation was non-significant, $r = -0{\cdot}21 (0{\cdot}05 < P < 0{\cdot}1)$. The results indicate a tendency for the basal metabolic rate to be negatively associated with length of stay in the tropics, but, since the groups comprise different individuals, not the same ones at different times, there is no positive evidence that the B.M.R. of an individual falls with duration of tropical residence.

The difference in level of significance between the results with the Aub-DuBois and with the Boothby-Berkson-Dunn standards can only be accounted for by a difference in the age factor in the two standards, the subjects in group 4 (tropical service 24–36 months) being on the average about 4 years older than the others. The basal values of systolic blood pressure, rectal temperature and pulse rate in relation to length of residence in the tropics, are also included in Table 3. None of those functions showed a significant correlation with time in the tropics.

The possibility of some deterioration of physical fitness occurring during an extended stay in the tropics, was investigated in a group of fifty-three British physical training instructors. They initially performed both the pack test and the physical fitness ratio test, and a week later twenty-seven of the men again

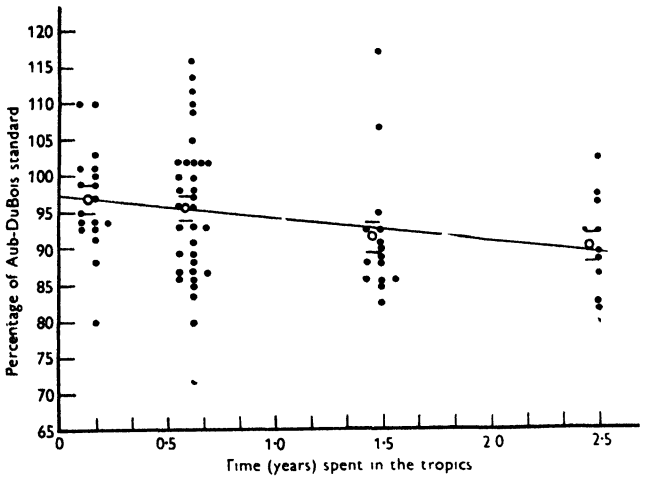

Fig. 1. The relation between B.M.R. and length of tropical service in British airmen. The unfilled circles indicate the mean values of the B.M.R. for different lengths of stay and the short horizontal lines the standard error of the mean in each case. The equation for the regression line is $y = 97{\cdot}21 - 2{\cdot}93x$.

TABLE 3. Physiological characteristics of groups of British airmen with different lengths of tropical service. Group means and standard errors are given.

Numbers of observations are given in brackets

Months in the tropics...	1–3	3–12	12–24	24–36
B.M.R. (Aub-DuBois standard)	96·8 ±2·0 (18)	95·6 ±1·8 (34)	91·9 ±2·2 (15)	90·4 ±2·0 (11)
B.M.R. (Boothby-Berkson-Dunn standard	94·7 ±2·0 (18)	93·8 ±1·8 (34)	90·5 ±2·2 (15)	89·7 ±2·0 (11)
Systolic blood pressure (mm. Hg)	117·0 ±2·0 (18)	114·0 ±1·6 (32)	113·5 ±3·5 (13)	111·6 ±2·8 (11)
Pulse rate per min.	64·0 ±2·1 (18)	66·0 ±1·6 (41)	63·0 ±2·4 (13)	60·0 ±2·5 (11)
Rectal temperature (° F.)	98·11±0·10 (18)	98·45±0·07 (41)	98·12±0·15 (15)	98·23±0·14 (10)

performed the latter test. The correlation between the scores on the first and second physical fitness ratio test was +0·86. With neither type of test was there a significant correlation between the fitness scores and the time spent in the tropics. This would suggest that physically trained men after 4 years in the

tropics are as fit to perform tasks involving hard work or requiring agility as they were 6 months after arriving in the tropics. Table 4 shows the mean score of this group on the pack test compared with those of other British and Indians in other occupational groups. The fitness scores of the British, except in the case of the hill porter, are always higher than those of the Indians in corresponding groups.

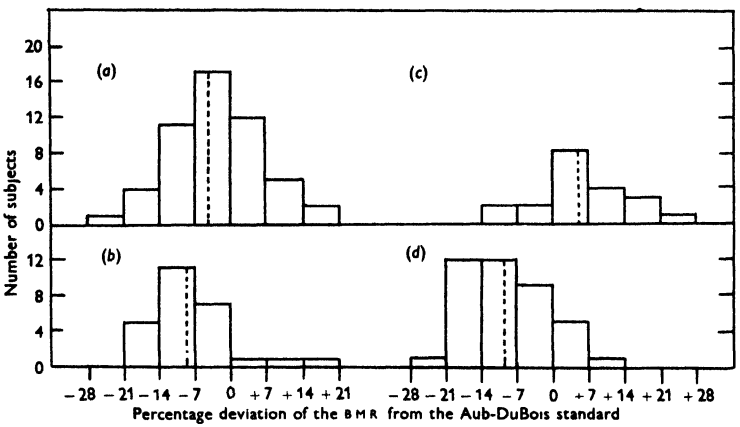

Fig. 2. Frequency distributions of the B.M.R. deviations from the Aub-DuBois standards in (*a*) British airmen after 1–12 months in the tropics, (*b*) British airmen after 1–3 years in the tropics, (*c*) British airmen in Kashmir in winter, (*d*) Indian airmen in the tropics. The dotted lines indicate the arithmetic means.

Indian subjects

Table 1 shows that the group had a lower B.M.R. than the British group described previously, the deviation from the Aub-DuBois standard being −9·6% and from the Boothby-Berkson-Dunn standard, −11·4%. The frequency distribution of the Indian B.M.R.'s are shown in Fig. 2*d*. The mean systolic blood pressure is also significantly lower than in the corresponding British group (Table 1). There is a high proportion of the Indian subjects (Fig. 3) with systolic blood pressures that would probably be regarded as extremely low (80–90 mm.) by European or American standards, none of the British subjects even after long residence in the tropics having a systolic blood pressure lower than 90 mm. Pulse rates were on the average slightly but not significantly higher than in the British group, as were also the mouth temperatures (Table 1).

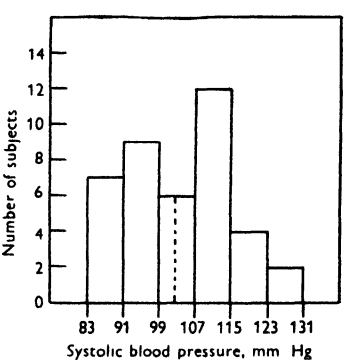

Fig. 3. Frequency distribution of the systolic blood pressure under basal conditions of forty Indian airmen. The position of the dotted line indicates the arithmetic mean.

A division of the data on the basis of the occupation of the subjects showed that the physical training instructors had a higher mean B.M.R. than the clerks, but the difference (3·7 %) is not significant. The mean score of the former on the pack test was, however, significantly higher ($P < 0{\cdot}01$) than that of the clerks (Table 4). Analysis of the data on the basis of the place of origin of the subjects showed little difference in either the mean B.M.R. or the score on the pack test of Indians from the north and south. In each of the three occupational groups, the Indians were less capable of performing the intense muscular effort demanded in the test than the British (Table 4). The difference is not probably due to the slighter build (Table 1) of the Indians since there was not a significant correlation between heaviness of build and fitness score in the group.

TABLE 4. Comparison of pack test scores of British and Indian airmen of similar occupation. Group means and standard errors are given

Description of group	No. in group	Time in tropics	Place of test	Score on pack test	Significance by t test
I. Skiing instructors (British)	3	1–2 yr.	Kashmir (8000 ft.)	115 ± 12·0	—
II. Hill porter (Kashmiri)	1	—	Kashmir (8000 ft.)	118	—
III. Physical training instructors:					
(1) British	53	6 mth.–4 yr.	Secunderabad (2000 ft.)	96 ± 1·6	III (1)–III (2) $P < 0{\cdot}001$
(2) Indian	21	—	Secunderabad (2000 ft.)	76 ± 3·8	
IV. Aircrew:					
(1) British	30	1–3 mth. in Poona	Poona (2000 ft.)	88 ± 1·6	IV (1)–IV (3) $P < 0.01$
(2) British	25	1–12 mth. in India	Kashmir (5000 ft.)	73 ± 2·0	
(3) Indian	21	—	Poona (2000 ft.)	80 ± 1·8	
V. Clerks:					
(1) British	20	6 mth.–3 yr.	Secunderabad (2000 ft.)	66 ± 3·8	V (1)–V (2) $P < 0{\cdot}02$
(2) Indian	19	—	Secunderabad (2000 ft.)	53 ± 3·0	

Effect of exposure to cold after residence in the tropics

Basal metabolism, blood pressure and pulse rate. The measurements were made to find out how men acclimatized to tropical heat reacted physiologically when transferred to a cold environment. During the journey from Bombay to Kashmir the change in environmental temperature was considerable, the air temperature in Bombay being about 80° F. and in Kashmir between 20 and 30° F. Indoor air temperature on the station could not be raised much above 30° F. at any time owing to lack of fuel, and the men were therefore continually dependent upon their clothing and their physiological reactions for warmth.

Three days after arrival at the mountain centre, that is about 5 days after initial exposure to cold weather, the basal metabolic rates and other values were measured. It would appear that 5 days of exposure to increasingly severe cold sufficed to raise the metabolism of the group significantly ($P < 0{\cdot}001$) above the tropical level (Table 5). Table 5 also shows that the British skiing instructors, who had been living in this region for about 6 months, had an average B.M.R. about 10% higher than the above group; and that the Kashmiri hill porters had still higher metabolic rates which averaged 31% above the mean value (Table 1) of the Indian group. The Kashmiris were not, however, under basal conditions, having had a light carbohydrate meal about 4 hr. before the estimation. They had been resting for half an hour before the measurements. The mean pulse rate of the visiting group was 70 ± 2 beats per min., which exceeds the value for British personnel in Bombay, and was probably an initial reaction to the increase in the altitude. There are no striking differences in the other measurements.

TABLE 5. Values of B.M.R., systolic pressure and pulse rate of British aircrew and Kashmiris, measured in temperate conditions after exposure to cold

Subjects			Boothby-Berkson-Dunn standard	Aub-DuBois standard	Systolic blood pressure (mm. Hg)	Pulse rate per min.
No.	Description	Time in Kashmir				
20	R.A.F. personnel from Poona	3 days in Srinagar Temp. 14–35° F., 5000 ft.	103 ± 2	105 ± 2	107 ± 2	70 ± 2
	Percentage deviation from mean tropical value (British)		+10	+12	-7	+10
3	Staff at Mountain Centre	6 mth. at Gulmarg.	115	117	95	49
		8000 ft. 1 mth.	113	115	110	55
		'Very Cold'. 4–14° F.	111	112	110	64
	Percentage deviation from mean tropical value (British)		+21	+22	-9	-11
3	Hill porters	Natives	112	112	95	60
			120	124	107	57
			119	124	102	68
	Percentage deviation from mean tropical value (Indian)		+31	+32	-2	-7

Pack test scores during and after Hill sojourn. Table 4 contains the scores of the above groups on the pack test. The aircrew who were tested 3 days after arriving in Kashmir are the only British group whose score falls below that of the Indian group of similar occupation. This is probably because they were performing the test at an altitude 3000 ft. higher than were the groups at Poona. But after another 3 weeks in the hills their mean score had increased significantly ($P < 0{\cdot}05$) to a value of 105 ± 18. Subsequently fifteen of the group were tested 10 days after their return to Poona and their mean score was found

to have dropped by 32 % to 87 ± 2, the fall being significant ($P < 0{\cdot}05$). Nine of the same men tested 1 month after their return to Poona had a mean score of 89 ± 2 which is not significantly different from the previous value. The initial improvement was probably due to acclimatization to the increased altitude and to exercise, whilst the relatively abrupt loss of the fitness which had been gained, was probably a result of re-exposure to the heat of the plains.

DISCUSSION

It would appear from the foregoing data that the basal metabolic rate of Europeans may change as a result of continued exposure to a hot or cold climate. Other investigators, however, who also have measured the B.M.R.'s of Europeans in the tropics, find that the response is not the same in all instances. Mason (1940) at Madras, and MacGregor & Loh (1941) at Singapore, measured the B.M.R.'s of the same persons at various times after their arrival in the tropics. Only in some was there a decrease, in others there was no change. According to Mason, the body temperature remained constant during tropical residence in the first type, but rose in the second type. In the present investigations there was an absence of correlation between rectal temperature and B.M.R.

It is not clear whether, when a fall in B.M.R. occurs, it is characteristically sudden or slow and progressive. Possibly the reaction varies in different persons. It would appear from the present data and those above mentioned that the decrease may continue at least for some months. On the other hand, Martin (1930), following the variation of his own B.M.R. during a journey to Australia, observed an abrupt diminution in level, in response to an extreme rise in air temperature. Increased muscular relaxation in the heat might be suspected in this instance as the cause of the fall. Krogh (1916) has suggested that climate may induce changes in the metabolic rate by influencing the muscular tone of the body. When the decrease is found to continue for a long period, it would seem more likely to arise from a gradual internal change; but the fact remains that there is at present no direct evidence to show how the diminution of metabolism is brought about in the human body. Hardy, Milhorat & DuBois (1941) describe an immediate chemical regulation of basal metabolic rate, occurring only in women, at air temperatures above 27° F. In animals, however, a regulation of heat production associated with endocrinal changes has been shown experimentally in response to change of climate (Ring, 1942; Horvath, Hitchcock & Hartman, 1938).

Other workers likewise have found low B.M.R.'s in Indians living in the tropics (Mason & Benedict, 1931; Mukherjee & Gupta, 1931; Wilson & Roy, 1938; Sokhey & Malandkar, 1939). The cause of this and also of the increase in basal metabolism which apparently occurred under the influence of climatic cold, are equally obscure. The latter increase accords with the few previous observations under somewhat similar conditions on Eskimos (Crile & Quiring,

1939) and on Mayas (Steggarda & Benedict, 1932). Manifest shivering did not occur in the subjects. Indeed they stated that they were comfortable during the tests; but changes in muscle tone for instance, occurring during the preceding exposure to the cold, may possibly have persisted during the test period.

It is interesting that, whilst the B.M.R.'s of the British subjects in the tropics tended in time to approach the Indian level, the blood pressure and fitness scores remained higher. The absence of a progressive diminution in the fitness scores of a group of British subjects with increasing length of tropical service was somewhat unexpected in view of the widely held opinion among all ranks of the service in the East that they felt less fit after long service there. Further investigation of this important point is clearly required before any definite conclusion can be reached. The present data, for instance, do not cover the first 6 months of sojourn when adaptation to tropical conditions generally is most likely to occur. Furthermore, selection factors may possibly have obscured a trend in fitness.

No reference has been found to other observations on the physical fitness of Indians, with which the present data may be compared; and information is equally lacking on other natives of hot climates. It would be of interest to know whether a smaller capacity than that of Europeans, for muscular work, is general. Jokl (1944), going on the athletic performance of American negroes, is of the opinion that the latter are potentially fitter than white people. Had the identity of environment existed for the British and Indians of the present tests, over the preceding years instead of months, the result might have been different.

SUMMARY

1. Groups of British and Indian airmen were compared in respect of their B.M.R., blood pressure, pulse rate, body temperature and score on the Harvard Pack test, whilst living under similar service conditions in tropical India.

2. The B.M.R. of the Indian group was 9·6% below the Aub-DuBois standard, whilst that of the British group, which had been in the tropics for 10·7 months was 5·6% below the Aub-DuBois standard.

3. The basal metabolism of the British group, which had been 3 years in the tropics, was close in value to that of the Indian group.

4. A few days of exposure to a cold hill climate was sufficient to raise the basal metabolism of a group of British subjects significantly above the tropical level.

5. The systolic blood pressure of the British group in the tropics was significantly higher than that of the Indian group. There was no significant tendency for the systolic blood pressure of the British subjects to fall with increasing tropical residence.

6. Neither pulse rate, rectal temperature, nor score on the pack test, showed any trend in value with continued tropical residence.

7. The pack test scores of the British groups were invariably higher on the average than those of the corresponding Indian groups, living and working under similar conditions.

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ON THE PREPARATION OF SECRETIN AND PANCREOZYMIN

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The existence in the mucosa of the small intestine of a material, distinct from secretin, which influences the external secretion of the pancreas, was first demonstrated by Harper & Raper (1943). They showed that secretin stimulated the production of water and bicarbonate only, and that the other material, which they named pancreozymin, increased the enzyme content of the pancreatic juice.

In their method of separating secretin and pancreozymin Harper & Raper followed closely the procedure adopted by Mellanby (1932) for the preparation of secretin, with the exception that they added a definite amount of bile salt to ensure complete precipitation of the secretin. The mucous membrane from the first metre of the small intestine of freshly killed pigs was extracted with alcohol. Calcium chloride was added to the extract, which was then filtered and concentrated to a quarter of its original volume. At this stage, to each 500 c.c. concentrate was added 1 g. bile salt (commercial tauroglycocholate) dissolved in 10 c.c. water followed by 15 c.c. 1% acetic acid. The precipitate obtained, containing the secretin, was separated in a centrifuge and purified by extraction with alcohol and re-precipitation with acetone.

The pancreozymin was not adsorbed on the bile acid precipitate but remained in the supernatant liquid. This was saturated with sodium chloride and allowed to stand in the dark at room temperature for 2–3 days. The sticky precipitate which separated contained the pancreozymin. It was dissolved in a small quantity of water and allowed to dry in a vacuum desiccator. This 'NaCl precipitate' was extracted with absolute alcohol and the material thus obtained was called the 'alcohol-soluble' preparation.

The bile salt used in these experiments came from a bottle of commercial tauroglycocholate in the laboratory and during the 3 years that this supply

lasted no difficulty was experienced in separating secretin and pancreozymin. Later, when new commercial samples of bile salts were used, low and variable yields of secretin were obtained. Appreciable amounts of secretin remained in solution and were precipitated with the pancreozymin when sodium chloride was added.

The unreliability of the method and the lack of uniformity in these results was attributed to variation in the composition of commercial samples of bile salts. In his earlier method of secretin preparation Mellanby (1928) added bile salt at one stage. He was aware of the variability of bile salt preparations but claimed that his method was adequate for 'all commercial preparations of bile, which, when dissolved in water, became opalescent on adding acetic acid to the extent of 0·1 %'. He later (1932) modified his method, and merely acidified the concentrated intestinal extract in order to obtain a precipitate, thus avoiding the addition of bile salts. The success of this method, however, seems to depend on the very variable amount of bile present in the intestine. If the intestinal mucosa is washed before extracting with alcohol the amount of precipitate at the stage of acidification with acetic acid is greatly reduced and the yield of secretin correspondingly low. It was for this reason that Harper & Raper added a definite amount of bile salt before acidification of the extract.

Difficulties with commercial bile salt preparations have also been encountered by the American workers Mortimer & Ivy (1929). They followed Mellanby's (1928) description, with the exception that they added 'Merck's technical sodium glycocholate'. They found that the method gave inconsistent results. Still (1931), who also attempted unsuccessfully to repeat Mellanby's experiments, suggested that the poor yields by this method were due to the use of alcohol as the agent for extraction of the mucosa.

It was obvious that a modification of Harper & Raper's original procedure was desirable. An investigation of the conditions for the adsorption of secretin by bile acids and a revised method for the preparation of secretin and pancreozymin were required. It was to these ends that the following experiments were directed.

METHODS

All the preparations were tested on cats which had been fed a few hours previously. The animals were anaesthetized with chloralose (0·075 g./kg. body weight intravenously). The splanchnic nerves were cut extraperitoneally and the pylorus occluded. The pancreatic duct was cannulated, the ligature passing round the bile and pancreatic ducts so that no further bile could enter the duodenum.

A continuous flow of pancreatic juice was maintained by injections of secretin intravenously at intervals of 12 min. in amounts sufficient to give a flow of 1·0–1·2 c.c./12 min. At intervals doses of the pancreozymin to be tested were given, together with the 'background' secretin. An injection of pancreozymin was always followed by two separate control doses of secretin alone before another injection of pancreozymin was given. The injections of secretin were given over a period of 10–20 sec. and the pancreozymin injections in 2 min. The amylase content of successive samples of pancreatic juice was measured by Wohlgemuth's method and the minute output of amylase, $D(J/T)$, calculated. The methods used were described by Harper & Vass (1941).

Bile salts were prepared from ox and pig bile by a slight modification of the method described by Cole (1933). 100 c.c. bile and 25 g. 'norite' charcoal were mixed and evaporated to dryness on a water-bath. The dry residue was extracted with 'methylated spirit' (95% ethyl alcohol, 4% methyl alcohol) in the proportion of 175 c.c. alcohol to 100 c.c. bile, on a boiling water-bath for 20 min. After cooling, the suspension was filtered through paper pulp and two volumes of ether added to the filtrate. The curdy white precipitate was allowed to settle overnight at 0° C. The supernatant liquid was then siphoned off and the residue separated by centrifuging. The precipitate was washed several times with ether and dried in a desiccator. From 100 c.c. ox bile the average yield of bile salts by this method was 2·14 g. A heavier average yield, 3·6 g., was given by the same amount of pig bile.

RESULTS

Secretin and pancreozymin were prepared by various modifications of Harper & Raper's orginal method. As a basis for comparison of the different secretin preparations 1 unit of secretin was defined as the activity present in 0·1 mg. of an arbitrarily chosen standard preparation. In a number of experiments the average amount of pancreatic juice secreted in the 12 min. period following intravenous injection of 0·1 mg. of this preparation was 1·2 c.c. In assaying samples of secretin, the preparations being tested were injected in doses which stimulated a flow of juice similar in volume to those produced in that particular experiment by injections of the standard preparation. By this method, the potency of any secretin fraction could be measured in units per mg. By combining this measurement of potency with the weight of the preparation per 100 g. of wet mucosa scraped from the intestine, the yield of secretin could be calculated as units per 100 g. of mucosa: e.g. in a fraction with a potency of 7·8 units/mg. and in which 57 mg. had been obtained per 100 g. mucosa, the total yield of secretin was $7{\cdot}8 \times 57 = 445$ units/100 g. mucosa. As the activity of secretin preparations gradually diminishes even when they are kept in the form of a dry powder, freshly made preparations were from time to time adopted as the standard. At each change over to a new standard the activities of the new and old standards were carefully compared and a conversion factor worked out so that the activity of all the preparations tested throughout the investigation could be expressed in terms of the original standard.

At the same time an attempt was made to gauge roughly the amount of any residual 'secretin activity' present in samples of pancreozymin, as indicated by the slight increase in volume of juice secreted in response to a combined secretin and pancreozymin injection, compared with the response to the same amount of secretin alone. The amounts of juice produced by the pancreozymin were very much less than those produced by injections of the standard secretin, but it was assumed that the amounts of juice would be proportional to the amounts of 'secretin activity'. Combining such measurements with the weight of pancreozymin preparations per 100 g. of intestinal mucosa an estimate could be made of the total amount of 'secretin activity' in any pancreozymin extract.

In comparing the pancreozymin activity of different preparations 1 unit of pancreozymin was defined as the activity of 1 mg. of an arbitrarily chosen

standard preparation. Five mg. of the standard was the yield from 3·8 g. of intestinal mucosa (wet weight). The preparations to be tested were injected in doses equivalent to 3·8 g. of intestinal mucosa, and the increase in enzyme output in the following 12 min. compared with the increases produced by injections of the standard preparation at the beginning and at the end of the experiment. As pancreozymin preparations, like secretin, show a gradual loss of activity even in the dry state, freshly made preparations were from time to time adopted as the standard and their activity related to that of the original standard.

When either pig or ox bile salts were used alone for preparing secretin and pancreozymin by Harper & Raper's (1943) method, an unsatisfactory separation of the two materials was obtained. With pig bile salts a thick curdy precipitate appeared when the secretin-pancreozymin concentrate was brought to pH 4·2–4·3 with acetic acid. On the other hand, with ox bile salts, the slight precipitate which appeared during concentration disappeared on the addition of the bile salt and the solution became clearer. Commercial samples of bile salts gave variable results with low yields of secretin, much being left behind with the pancreozymin.

Observations were made on the solubility at different pH's of mixtures of ox and pig bile salts in the ratio of 600 mg. ox bile salt to 200 mg. pig bile salt per 500 c.c. of solution (the proportion finally used to precipitate secretin). In these experiments all the various samples of ox and pig bile salts used in preparing secretin and pancreozymin were tested. It was found that the solubility varied with the composition of the buffer solutions used. Citrate and phenylacetate ions seemed to increase the solubility of cholic and other related acids, so that no precipitation of bile acids occurred in buffer solutions containing these ions even when the pH was as low as 3·4. On the other hand, when different pig and ox bile salts combined in the same proportions were added to a series of acetic acid/acetate buffer solutions, differing by 0·05 of a pH unit (checked by the glass electrode) it was found that all were precipitated to the same extent at a pH of 3·85, although they remained in solution at pH 3·95. It was also found that when peptone was added to the bile salt preparations, precipitation occurred in all cases at pH 4·05, both with acetic acid/acetate buffer and with acetic acid alone. This suggests that protein derivatives in the solution obtained by the extraction of the mucosa may also facilitate precipitation of the bile acids.

It seemed clear that to ensure a satisfactory separation of secretin and pancreozymin a very fine bile acid precipitate with a large adsorptive surface was required. By trying various mixtures of pig and ox bile salts a method was finally evolved whereby about 95% of the total secretin could be precipitated on adding a mixture of pig and ox bile salts. Any residual secretin was removed by the addition of more bile salts. The total amount added was 1 g./500 c.c.

concentrate, i.e. the amount originally prescribed by Harper & Raper. When larger amounts, up to 1·5 g./500 c.c., were used, no better separation of the two materials was obtained. The pancreozymin remained in the supernatant liquid and was precipitated when the solution was saturated with sodium chloride.

The following is a detailed description of the method. The mucous membrane from the first metre of the small intestines of freshly killed pigs is scraped off, ground with sand and extracted for 30 min. with four times its weight, expressed in c.c., of 'methylated spirit' (95 % ethyl alcohol, 4 % methyl alcohol). At this and all subsequent stages the material is protected from the light. The extract is filtered and stored in the ice chest until it is to be concentrated. Before concentration an equal volume of 0·1 M-$CaCl_2$ solution is added and the mixture allowed to stand for 15 min. It is filtered under slight suction through paper pulp and the filtrate is concentrated in 500 c.c. portions to a quarter of its original volume. The concentration is carried out by distillation under reduced pressure in the shortest possible time, i.e. $1\frac{1}{2}$–$2\frac{1}{2}$ hr. The temperature of the water-bath is controlled at 40° C.

A mixture of 600 mg. ox and 200 mg. pig bile salts, dissolved in 10 c.c. water, is added to each 500 c.c. concentrate followed by sufficient 2 % acetic acid (usually about 20 c.c.) to bring the pH to 4·0–4·2, measured by the glass electrode. The mixture is allowed to stand for 10–15 min. and precipitate I, containing most of the secretin, separated in a centrifuge. At this stage some secretin remains in solution. A 10 % solution of a mixture of bile salts is added in the proportion of 67 mg. ox and 133 mg. pig bile salt per 500 c.c. concentrate. (Precipitate II thus obtained may be treated as precipitate I if it is desired to assay its secretin content, or, since its potency is low, it may in routine preparations be discarded.)

Precipitate I is extracted with absolute alcohol. The alcoholic solution is spun off and the active secretin precipitated by the addition of four volumes of acetone. The precipitate is separated in a centrifuge, washed with acetone and dried in a desiccator over sulphuric acid.

The supernatant liquid from precipitate II, now almost free from secretin, is poured into a conical flask and saturated with NaCl (30 g./100 c.c.). It is allowed to stand 2–3 days at room temperature in the dark while a sticky precipitate settles out. From this the 'NaCl precipitate' is prepared by dissolving the precipitate in the minimum amount of water and evaporating to dryness in a vacuum desiccator over H_2SO_4. When this is dry it is extracted by shaking for 30 min. at room temperature with absolute alcohol (50 c.c./l.g.). The residue is separated in a centrifuge and the alcoholic solution evaporated to dryness in a vacuum desiccator. This is the 'alcohol-soluble' preparation which contains the pancreozymin. It is almost free from secretin.

The potency and yield of secretin from ten preparations made by this method were estimated, and the results are set out in Table 1. Weights of secretin and

pancreozymin per 100 g. wet mucosa varied considerably; the variation in the ten secretin preparations (precipitate I) was from 34 to 109 mg. (average 62 mg.), and in twelve pancreozymin preparations from 112 to 236 mg. (average 179 mg.). This variation may be due to differences in the composition of the bile salts used in the preparations, and in the amount of bile salt initially present in the mucosa before extraction.

TABLE 1. The potency, yield and percentage distribution of secretin in ten preparations

	Potency (units/mg.)	Yield (units/100 g. mucosa)		Percentage distribution
Secretin, precipitate I	6·8 (4·1–10·0)	380 (289–450)	402 (294–476)	94·5 (88·5–98·5)
Secretin, precipitate II	0·8 (0–2·3)	18 (0–50)		4·5 (0–11)
Pancreozymin	—	4 (0–8·5)		1·0 (0–2)

The figures in brackets indicate the range of results.

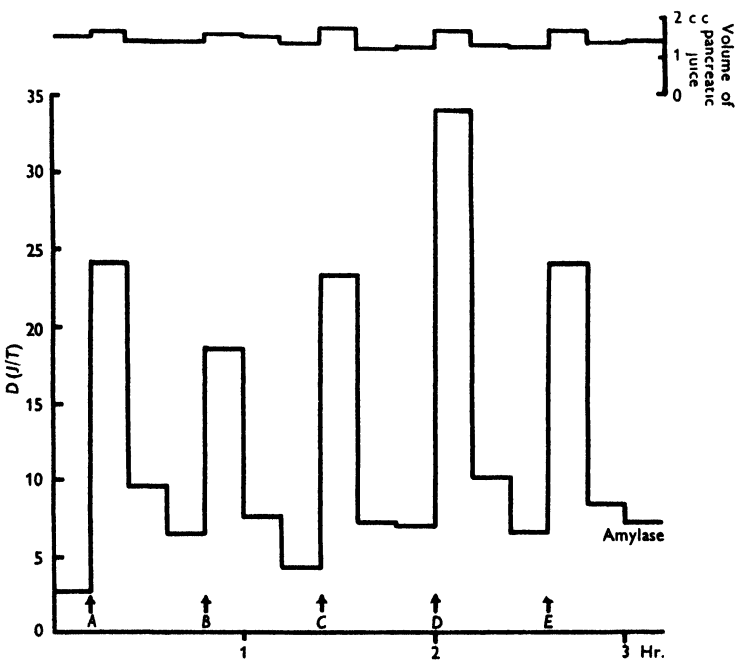

Fig. 1. Pancreatic secretion was maintained by injections of secretin at 12 min. intervals. Injections of the standard pancreozymin in amounts equivalent to 3·8 g. mucosa were given at *A* and *E*. Injections of the test pancreozymin were given at *B*, *C* and *D*. At *B* 3·8 mg. (=1·9 g. mucosa) were injected; at *C*, 7·6 mg. (=3·8 g. mucosa); and at *D*, 15·2 mg. (=7·6 g. mucosa). The increase in enzyme output following the injection at *C* is equal to that produced by the injections of the standard pancreozymin.

In testing the pancreozymin preparations it was found that there was a reasonably constant yield of pancreozymin when samples were injected equivalent

in terms of g. of wet mucosa to the doses of the standard preparation, i.e. 3·8 g. mucosa (Fig. 1). The weights of the different pancreozymin preparations of course varied considerably. In twenty-nine such tests on twelve pancreozymin preparations the average minute output of amylase in the 12 min. period after injection was 14·2 compared with an average minute output in the preceding 12 min. control period of 4·3. Such results were obtained only if the amounts of residual 'secretin activity' present in the pancreozymin was small. If the separation of the secretin and pancreozymin had been inadequate there was an apparently greater activity in the pancreozymin preparations, as the increase in volume of juice produced by the secretin increased the calculated minute output of amylase, e.g. in twelve tests on three earlier preparations which contained appreciable amounts of secretin there was an average post-pancreozymin amylase output of 32·0, compared with an average output of 5·0 in the preceding 12 min. period. The yield of pancreozymin per 100 g. of mucosa was about 130 units.

To compare the potency and yield of secretin by our modified method with that obtained by a method which did not involve the addition of bile salts, one batch of secretin was prepared by the S1 method of Greengard & Ivy (1938). In order to make the results comparable with our method the first metre of ten intestines was used for the experiment instead of the first 6 ft. described by Greengard & Ivy. In five tests the potency of this S1 secretin was found to be 2 units per mg. The total amount of S1 obtained from the ten lengths of intestine was 292 mg. The mucosa is not scraped from the intestine in Greengard & Ivy's method, but assuming the average amount of mucosa per intestine (40 g.) this would give a yield of secretin by the S1 method of 146 units per 100 g. mucosa.

DISCUSSION

Now that a satisfactory procedure has been worked out for the separation of secretin and pancreozymin it is of interest to note the differences in behaviour between various bile-salt preparations. It seems from our experiments that it is important that the bile acids from both pig and ox bile should be present in the mixture used for precipitation. Ox bile contains principally cholic acid conjugated with glycine and taurine, while the chief bile acid in pig bile is hyodesoxycholic acid conjugated with glycine. There is little or no taurine in pig bile. The presence of the sulphonic acid group of taurine in ox bile salts probably explains their greater solubility in acid solutions. It has been stated by Hammarsten (1895) that taurocholates will keep glycocholates in solution at low pH. This property may be an attribute of the taurine-conjugated acid and may be its only function in this precipitation, where it prevents the pig bile acid from forming an immediate coarse precipitate with poor adsorptive properties.

In their original experiments Harper & Raper were fortunate in having a commercial sample of bile salt available which apparently contained the

right proportions of ox and pig bile acids. Later, during the war, British commercial preparations of bile may have been manufactured almost exclusively from ox bile, since our pig population was so greatly reduced. It is possible that Still (1931) and Mortimer & Ivy (1929) had similarly prepared bile salts. Failure to get good yields of secretin in all these cases may have been due to lack of the pig bile acid. Mellanby's criterion for the effectiveness of bile salt in his (1928) method of preparation is obviously valueless. We have found that several commercial preparations which on this standard should have been effective in precipitating secretin gave poor yields and left much of the secretin in solution with the pancreozymin. Our own experiments have shown that the solubility of bile salts at various pH's is of little value at present in determining their effectiveness in the precipitation of secretin.

The modified method of preparation described above has been used with different batches of ox and pig bile salts prepared by ourselves. In all cases good separation has been obtained. Secretin and pancreozymin prepared by this method have been found to be sufficiently potent for use in human subjects.

The standardization of secretin and pancreozymin has recently been discussed by Burn & Holton (1948). The methods suggested by Ivy and his colleagues for standardizing secretin (Ivy, Kloster, Drewyer & Leuth, 1930), and pancreozymin (Greengard & Ivy, 1945) are straightforward measurements of the volume and enzyme content respectively of pancreatic juice, without reference to the activity of a standard preparation of secretin or pancreozymin. Still (1931) and Wilander & Ågren (1932), on the other hand, express the activity of their secretin preparations in terms of the activity of a standard preparation.

The main difficulty in measuring the activity of secretin and pancreozymin by their effects on the output of juice or of enzymes is the great variability in the response of the pancreas in different animals to these materials. In acute experiments this variability may be due to the effects of the anaesthetic, to the amount of operative trauma in preparing the animal, to variations in the previous diet of the animal or to other unrecognized factors. To eliminate this source of error preparations would have to be tested on a number of animals and an average response obtained; a procedure which would be wasteful and impracticable when large numbers of preparations have to be compared. For this reason, in the present investigation, we adopted the measurement of secretin and pancreozymin activity in terms of arbitrarily chosen standards.

Another difficulty in measuring the activity of secretin and pancreozymin is that the responsiveness of the pancreas may vary in a single experiment in the course of a few hours, the variation usually being a gradual decrease in responsiveness. To minimize this source of error we have compared test preparations of secretin with injections of standard secretin given 12 min. before or 12 min. after the test preparation, and injections of standard pancreozymin have been given at the beginning and end of the experiment so that any

decrease in responsiveness might be apparent. The gradual diminution in responsiveness of the pancreas may be due to the preparation of the animal in these experiments. The animals were routinely fed before the experiment, and the splanchnic nerves were cut extraperitoneally before the abdomen was opened. This ensured in most experiments that the pancreas was very responsive to both secretin and pancreozymin, but the responsiveness did tend to decrease in many experiments during the period of injection. In other experiments in the laboratory on fasting animals in which the splanchnic nerves have not been cut, the pancreas has been found to be less responsive to secretin but the responsiveness has varied little over 4 or 5 hr. For the routine assaying of secretin and pancreozymin it might be advisable to use the less sensitive but also less variable fasting animal with the splanchnic nerves intact.

Measuring secretin and pancreozymin activity in terms of a standard preparation is made more difficult by the gradual loss of potency of dry preparations of secretin and pancreozymin, which becomes obvious after a year or two. This difficulty, which has been experienced also by Lagerlöf (1942) with secretin, was avoided by changing to freshly made standards every few months. It would be very useful to have standard preparations of secretin and pancreozymin which maintained their potency indefinitely. Burn & Holton suggest that their acetone-dried powders may fulfil this requirement.

It was suggested by Still (1931) that the yield of secretin was small if alcohol was used for the extraction of the intestinal mucosa. In our hands an extraction with N/10 HCl (S1 preparation of Greengard & Ivy) gave a less potent secretin preparation and a smaller total yield of secretin than extraction with alcohol and adsorption of secretin on a bile acid precipitate. Our unfamiliarity with the S1 method of preparation probably explains the poor yield obtained, but the result suggests that alcohol is at least as effective as N/10 HCl in extracting secretin.

SUMMARY

1. Inconsistent results which have been encountered in the original method of Harper & Raper for the separation of pancreozymin and secretin have been investigated, and found to be due to the nature of the bile salts originally used in the process.

2. A modified method of preparing secretin and pancreozymin, sufficiently potent for use in man, is described. Secretin and pancreozymin are extracted from the intestinal mucosa by alcohol. The secretin is separated from the pancreozymin by adsorption on a precipitate of mixed pig and ox bile acids, and the pancreozymin precipitated from the supernatant fluid by saturation with NaCl.

3. The conditions governing the adsorption of secretin on bile acid precipitates have been investigated.

4. 'Units' of secretin and pancreozymin are defined, and the assaying of secretin and pancreozymin is discussed.

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THE MONOCHROMATISM OF THE CENTRAL FOVEA IN RED-GREEN-BLIND SUBJECTS

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The tritanopia of the central fovea of the normal subject was first described by König in 1894, but the observation passed relatively unheeded. This happened very largely because of an unfortunate confusion in nomenclature and through a misunderstanding of the unsatisfactory term 'blue-blind'. Perhaps, also, the observation was allowed to fall into oblivion because it was difficult to reconcile with the part which cones were supposed to play in the perception of colour, according to the current trichromatic theory; for, if cones were the only elements responsible for colour discrimination and three types were required in order to satisfy the data from experiments on colour mixing, then it was difficult to see how an area believed to consist entirely of cones should not have the complete trichromatic mechanism. König's detailed account, however, was completely and independently confirmed a few years ago (Willmer, 1944; Willmer & Wright, 1945) when it was found that the errors which are made in matching colours on very small fields were not random errors but followed a systematic pattern, and were, in fact, practically identical with those which have been described as characteristic of the tritanope (Pitt, 1944); moreover, it was again shown that the normal subject could match all the spectral colours with mixtures of red and violet, provided that the fields fell within the central foveal area and that central fixation was maintained.

For most normal observers, fields, which subtend about 1° at the eye and are centrally fixated, theoretically fall within the so-called 'rod-free area' of the fovea. In practice, however, three factors make it desirable to use a considerably smaller field than this in investigations of the behaviour of this area. In the first place, recent observations (Lord & Wright, 1948; Barlow, 1949) have shown that small eye movements frequently occur, and these may be sufficiently large to bring margins of the 1° field on to parts of the retina outside the central fovea. Secondly, the field size should be kept small and its intensity low, because sufficient light may otherwise spread to neighbouring areas and cause stimulation of extra-foveal receptors. In the dark-adapted eye, for example,

the low-threshold rods may be about one thousand times more sensitive than the elements in the central fovea, so that a very small amount of scatter from the central spot may involve these receptors also. Thirdly, there may be some lateral spread of excitation within the retina itself and this should not be allowed to reach the extra-foveal elements. It may be difficult, or in some cases even impossible, to prevent the surrounding areas from becoming involved, but the smaller the field and the more carefully it is centrally fixated the less interference is likely to be caused. The ganglion cells which are stimulated by the rods 'cover' relatively large areas of the retina and their shape and connexions make it not unlikely that their behaviour may be determined by other elements besides the low-threshold rods. Clearly, then, it is very important in investigations of the properties of the central fovea to use considerably smaller fields than the dimensions of the so-called rod-free area suggest. Preferably such fields should not subtend more than 30′ at the eye. Moreover, Thomson & Wright (1947) have shown that the foveal area is not uniform, and that progressive differences in apparent hue occur as a small (15′) field is moved from the position of central fixation to positions 20′ and 40′ eccentrically.

When a normal subject examines a divided field, or two small separate fields, with his central fovea he may at first notice nothing curious about the colours subjectively, and he may be able to distinguish between colours in apparently the normal way. As he becomes firmly fixated, however, and after a measurable delay, which gets less with practice, then the subjective impressions change, and, what is more important, he makes definite confusions.

Even under these conditions, however, the normal subject can *subjectively* distinguish a very wide variety of colours with his central fovea, though his hue discrimination is very much reduced (Willmer & Wright, 1945). Red and blue-green are the colours most characteristically registered by the normal central fovea, though a curious saturated violet is also seen. Yellow tends to be indistinguishable from white and may be called either yellow or white. Similarly, violet, blue and blue-green are all confused to some extent. A bright violet may appear to be blue or blue-green, though the converse does not seem to be true, i.e. when a blue-green (say $\lambda = 480$ mμ.) is reduced in intensity it does not appear violet but tends rather to a greyish hue. However, these subjective impressions are open to all manner of suggestions and are notoriously unreliable; the main *objective* fact about the normal central fovea is that, within that area, all spectral colours can be matched by mixtures from two colours only, provided that these are chosen from the spectral extremes. The mixtures are specific for each wave-length and the matches only hold within definite limits. This suggests the presence of only two separate pathways, with different spectral sensitivities, between the retina and the brain. Moreover, the fact that particular mixtures of the spectral extremes match particular wave-lengths shows that the matches are genuine matches and not random errors made by

the subject because the matching fields are too small to allow of precise judgement. Furthermore, it may be concluded that no more than two pathways are involved, for, if they were, then the matches and confusions which are characteristic of the central fovea would not be made, since the third or other pathways would give the information required for distinguishing the colours. For example, a mixture of red (R) and violet (V) can be used to match green (G) in the central fovea because green in that area is seen when receptor A responds with intensity a and receptor B with intensity b (Fig. 1). Wave-lengths V, stimulating receptor A to the extent v, and R, stimulating receptor B to the extent r, can thus be chosen and used in sufficient intensities to stimulate receptor A to the extent a and receptor B to the extent b. Thus $vx=a$ and $ry=b$. If, however, a third receptor C were involved, whose spectral sensitivity was different from

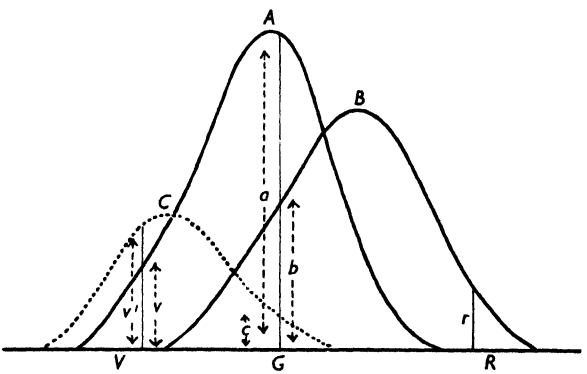

Fig. 1. Diagram to illustrate the difference between dichromatic vision and trichromatic vision. A and B, sensitivity curves (hypothetical) for the two receptors present in the central fovea; C, sensitivity curve, also hypothetical, for a third receptor. V, violet; G, green; R, red; a, sensitivity of receptor A to green; b, of receptor B to green; c, of receptor C to green; v, of receptor A to violet; v', of receptor C to violet; r, of receptor B to red.

those of A and B and was not uniform through the spectrum, then wave-length V would stimulate it to the extent v' and it would be extremely unlikely that, if $vx=a$, $v'x$ should also equal c, where c represents the extent to which receptor C is stimulated by wave-length G when this wave-length is stimulating receptors A and B to the extents a and b respectively. It is clear, therefore, that if more than two receptors are involved it would be almost impossible to make matches with all the spectral colours by means of two primary colours only.

The central fovea of the normal subject therefore behaves as though it possessed two independent pathways, and there are very strong indications that it has only two pathways. It does not follow that structurally there are only two pathways, though the evidence for this is strong, but, functionally, two pathways are the necessary and sufficient requirements to satisfy the data

with respect to colour matching, etc., in the central fovea, when such data are obtained under conditions which minimize the possibility for interaction from other retinal elements. In this connexion it may be again emphasized that the foveal matches described are only made after a perceptible lapse of time after the image has apparently been fixated. The reason for this delay is unknown, but it can be reduced by practice. It may be due to the interference by some third pathway being 'inhibited' out, or it may simply be necessary to wait till the information, derived from other parts of the retina during the process of fixation, has faded from memory before a pure central foveal observation can be made.

If, therefore, the normal subject has only two instead of three pathways from his central fovea and the missing path is the so-called 'blue path', then it is pertinent to inquire how the red-green-blind observer behaves with respect to his central fovea. He may be lacking his red receptor if he is a protanope, or, if he is a deuteranope he may lack his green receptor, or if Pitt's (1944) interpretation is correct, he may have his red and green receptors combined into a single pathway. At this point it must again be stressed that the 'blue' receptor does not mean the receptor which necessarily gives rise to the sensation 'blue', but it means the receptor which 'covers', more than the other two, the blue end of the spectrum. Similarly, the green receptor has its maximum in the green region of the spectrum, though it probably covers most of the visible range, and there is no evidence that greenness is the result of stimulation of this receptor alone. Red, green and blue receptors are the terms given to denote the regions of the spectrum in which the receptors have their maximum sensitivities and their stimulation should not be correlated with any particular sensation, for the latter are much more likely to arise as the result of different proportional responses from two or more of the receptors. The original failure to accept König's observations on the tritanopia of the central fovea was caused because he described the condition as one of blue-blindness, meaning absence of the receptor mechanism covering the blue end of the spectrum, and not the inability to have the sensation of blue aroused by stimulation of this area. The sensation of blue can be aroused from the central fovea, though this area is relatively insensitive to the blue end of the spectrum and behaves as if it were lacking a receptor covering this region.

A red-green-blind subject may therefore be expected to become totally colour-blind if his vision is restricted to the central fovea, because he has only one pathway where the normal person has two. Such an observer should be able to match any colour with any other colour provided that the intensities were also matched, and the following experiments describe a simple technique for showing that this is so.

METHODS

Essentially, the apparatus has been designed to illuminate two small fields, each subtending 10′ at the eye and with their centres separated by a distance equivalent to 20′, with light from Ilford

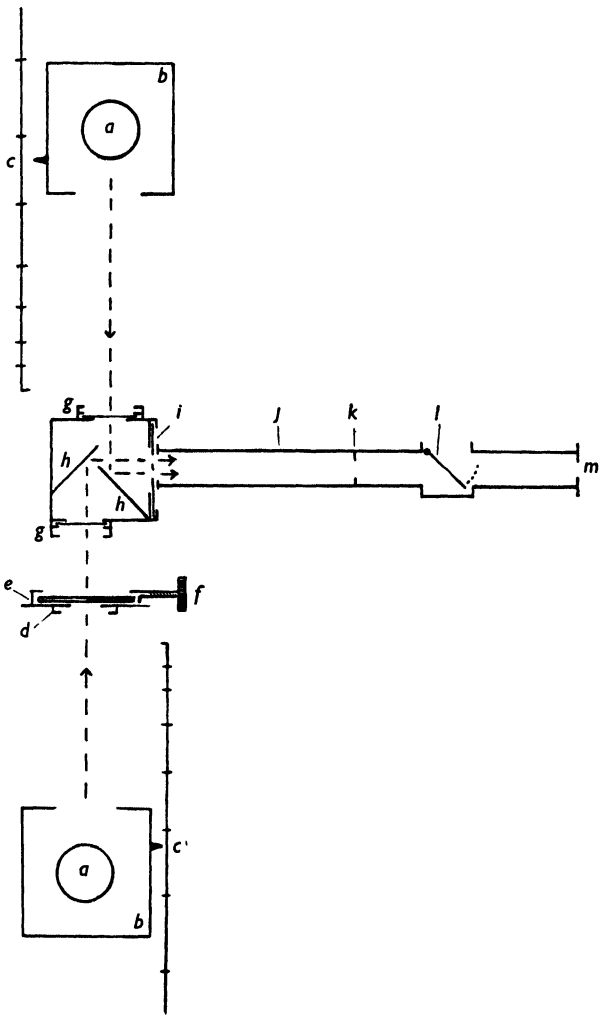

Fig. 2. Plan of apparatus (not to scale). *a*, 100 W. tungsten filament frosted lamp; *b*, lamp-house mounted on runners; *c*, scale of intensities, relative to unity at the end of the scale; *d*, filter-holder for neutral filters, etc., in front of 2·5 cm. aperture; *e*, holder for blue and red dipartite filter; *f*, micrometer screw with scale for moving dipartite filter across aperture; *g*, filter-holder and ground-glass screen; *h*, front-silvered mirrors; *i*, stop for regulating dimensions and shapes of viewing fields; *j*, viewing tube; *k*, stop for preventing internal reflexion; *l*, holder for mirror or glass plate which can be used to reflect fixation spots, adapting lights, etc., and which can swing out of line when not required; *m*, artificial pupil.

spectral filters. The left-hand field was illuminated by light or a single wave-band, obtained by using one of the Ilford filters, 601–609, whose intensity could be varied by the insertion of neutral

filters or by appropriate movements of a 100 W. tungsten lamp along a scale graduated in intensity units relative to the ground-glass screen which formed the basis of the visible field, and which was in a constant position relative to the eye. The right-hand field was illuminated by light from another similar 100 W. tungsten lamp passing through a divided filter of which one half was blue (Ilford 602) and the other red (Ilford 608), and falling on a second fixed ground-glass plate. This filter could be moved by a micrometer screw across the beam of light so that the small right-hand field could be illuminated with pure red light, pure blue light or with mixtures of the two in any proportion. The light falling on the ground-glass plate had passed through a square aperture of side 2 5 cm. and the micrometer scale indicated the number of millimetres of the aperture occupied by the red filter. Separating the red and blue filters was a narrow (1 mm.) band of black. The intensity of the mixed field could also be varied if necessary by neutral filters or by movement of the light source along a graduated scale. The light from the two ground-glass plates was reflected by front-silvered mirrors in such a way as to illuminate the two fields which were formed by means of two 2 mm. holes in a brass plate which was viewed from a distance of 70 cm. By the use of alternative brass plates as 'stops' various other field sizes up to and including two adjacent semicircular fields subtending 2° at the eye could be obtained. A 2 mm. artificial pupil was used throughout. It was placed at the end of a viewing tube fitted with a suitably placed stop to prevent internal reflexion along the tube, and the tube itself prevented interference by extraneous light. The general plan of the apparatus is shown in Fig. 2. Observations were made in a darkened room illuminated only by stray light from the apparatus which was reduced to a minimum by enclosing as much as possible of the apparatus within black screens. Readings were taken only after the subject had been in the dark for at least 10 min., by which time 'cone adaptation' is nearly complete. The subject was asked to fixate on the space between the two fields or on the left-hand edge of the right-hand field. He held his head relatively firm by biting on an adjustable dental impression clamped to the bench. Owing to the chromatic aberration of the eye, the light from the red and blue filters is apt to separate when the eye is not viewing the fields directly. The correct position of the head can therefore be checked by ensuring that the blue and red fields overlap on the right-hand small field. Observations were only made and readings taken when the subject considered that he was fixating the fields centrally. A blue filter was chosen rather than a violet since the pure violet filter transmits so little light that many of the matches would have to be made at a very low intensity, with consequent inaccuracy. The two filters did not transmit equal quantities of red and blue and in any case the relative brightness of the two filters was different for different observers, particularly if the latter were colour-blind. The scale readings for the amounts of red and violet light thus represent purely arbitrary values and it must be borne in mind that as the amount of red increases, so the amount of blue declines, and vice versa. The quantitative data for the amounts of red and blue which match other spectral colours for the normal central fovea have already been published (Willmer & Wright, 1945).

RESULTS

In preliminary experiments the subjects were asked simply to find the position of the dual filter so that the field illuminated by it matched in hue the other field which was illuminated with a single wave-band. The light source for the mixture of red and blue was maintained at a fixed position throughout the experiments. The brightness of the single field could be adjusted to match the brightness of the dual field. It was soon found that this method led to great variability in the readings obtained, and if adjustments were made to the brightness then several positions could be found over which a match could be obtained. In later experiments, therefore, the subject was asked to add as much red to the mixture as he could and still preserve a match for the single field which he was allowed to vary in brightness as much as he liked. Having

found the extreme red position he then tried to find the extreme blue position, again being allowed to vary the intensity of the single field. Between these two positions it was therefore possible to match the single field at some brightness level with mixtures of red and blue. Outside these limits it was impossible to make a match, however the intensity or proportions were altered.

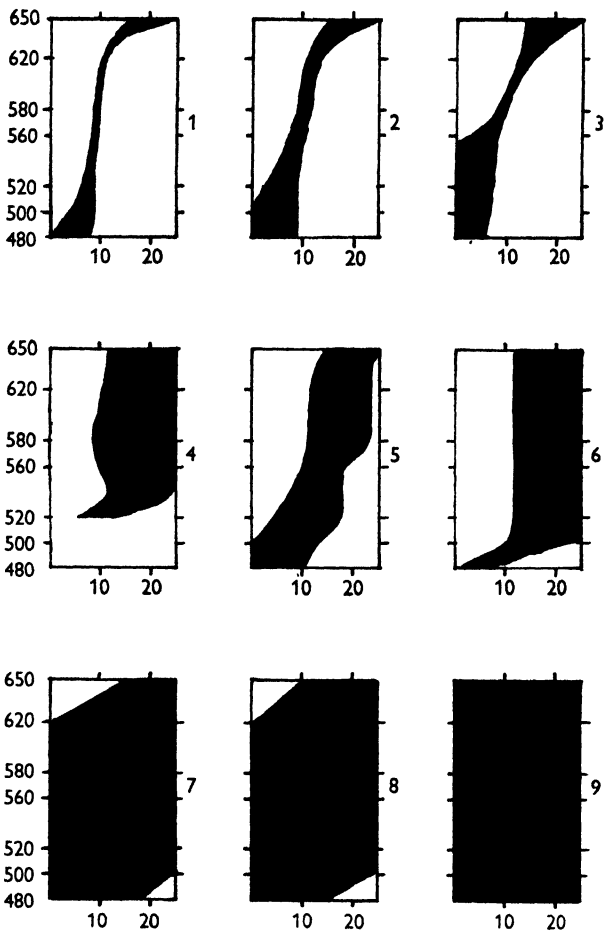

Fig. 3. Diagrams illustrating the ranges within which matches between spectral wave-bands and mixtures of red and blue lights are valid for normal, colour-anomalous and colour-blind subjects, when using two 10′ fields separated by 10′ and centrally fixated. Black areas indicate the range. 1, 2 and 3, normal subjects; 4–8, subjects with varying degrees of colour-blindness; 9, protanopes and deuteranopes. Ordinates, wave-lengths; abscissae, scale readings of dipartite filter position; 0 = blue; 25 = red.

When a normal subject matches the various wave-bands with mixtures of red and blue the results can be plotted as in Fig. 3. For each particular match there is a definite and often considerable range over which the match holds provided

that the subject is allowed to alter the intensity of the field to be matched. This range is least in the yellow region of the spectrum but progressively increases towards the spectral extremes. The width and the exact pattern of the area on the diagram which records the limits within which the match holds, vary somewhat from individual to individual but for normal subjects they are roughly the same (Fig. 3, subjects 1–3).

When some degree of colour anomaly is present, the pattern changes and probably assumes characteristic shapes according to the type of anomaly, though this has not, in fact, been tested systematically. With protanopes and deuteranopes, however, as these are determined by the Ishihara test or by their ability to match red and green on a 2° field, the pattern changes to a simple black rectangle because such subjects can match any colour with either red or blue or with any mixture of the two provided that central fixation is maintained and that they vary the intensity of the field to be matched.

In other words, protanopes and deuteranopes are completely colour-blind when their vision is confined to the central fovea. In this region they behave as monochromats and require only one primary colour, to match all other colours and also white.

Some subjects can match all the spectral colours between red and blue-green with red but they detect a difference between these colours and blue and, similarly, there have been found several subjects who can match red with all colours as far as blue but they detect violet as something different. The reason for this is not yet determined. It may be that the 'blue' receptor which is usually absent from or only very sparse in the central fovea may be present to a greater extent than usual in these subjects. Alternatively, it may be that such subjects have more difficulty than most in maintaining fixation with the requisite accuracy so that the match is therefore frequently 'breaking' because information is obtained from the foveal slopes or parafovea. Under these conditions the subject may not be able to separate the information obtained from the central fovea only, from that obtained from surrounding areas.

With regard to the normal subject and the subjects who have colour anomalies the results are clearly of a preliminary character. With a modification of the apparatus which would allow better control of the separate intensities of red and blue it should be possible to obtain important data on the relationship between intensity and hue discrimination. Since the normal fovea has only two pathways, experimental results obtained from this area alone should give data of a simpler kind than those derived from the usual triple system for the whole eye.

A few subjective observations may be noted. Most of the colour-blind subjects report the fields when matched as having lost all colour; others describe them as grey-blue; and in one case, which happened to be that of a deuteranope, who would, in earlier days, have been described as green-blind, all the spectral colours when matched on the central fovea were described as appearing green.

SUMMARY

1. A simple method is described for matching spectral colours, obtained by filters, with mixtures of red and blue, on small fields and with central fixation.

2. Provided that central fixation is maintained and that the intensities of the fields are properly adjusted, normal subjects show a characteristic range in the amounts of red and blue in the mixtures which match the particular wave-bands; colour-anomalous subjects show significant variations in this range; protanopes and deuteranopes can match all wave-bands with one primary only.

3. It is concluded that while the central fovea of the normal subject is dichromatic, that of protanopes and deuteranopes is monochromatic.

4. This monochromatism of the central fovea of red-green-blind subjects is thought to follow logically from the dichromatic character of the same area in the normal subject and suggests that a simple trichromatic theory is both necessary and sufficient to account for the facts of colour vision. It is unlikely that more than three types of receptor are involved, but it is possible that one or even both of the receptors of a diodic system (i.e. a system having two independent receptors and pathways) might show variations in sensitivity in different parts of the eye in such a way as to be capable of providing the necessary third variable.

I wish to express my great gratitude to all those who have given freely of their time and patience in order to make observations under somewhat exacting conditions.

A grant from the Leverhulme Trust has made possible the completion of this work and I wish also to express my appreciation of this assistance.

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IRON SUPPLIES IN FOETAL AND NEWBORN RATS

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The object of the experiments was to study iron supplies in the foetal rat with the help of tracer technique using Fe^{59}. The investigations were extended to cover the period of lactation.

METHODS

Female rats (140–200 g.), which on daily swabbing were found to have sperms in the vagina, were given a tracer dose of 0·1 mg. of labelled iron in the form of a subcutaneous injection of a solution of ferric ammonium citrate. Injected rats were placed in a separate cage, and if they subsequently proved to be non-pregnant they were used as controls.

No special measures were taken to control the diet or to induce a high uptake by the blood of the tracer dose, as it was desired to obtain, as far as possible, a picture of the normal state of affairs.

Rats were killed at regular intervals from the 14th day of pregnancy until 49 days post-partum, and specimens of maternal blood and whole foetuses were obtained for ashing. Specimens were dry ashed at 450–500° C. for 3 hr. The ash was dissolved in HCl, and an aliquot used for the colorimetric determination of the amount of iron using the thioglycollic acid method. To the remainder (for radioactive assay) was added 4 mg. of carrier iron, and the whole precipitated with 'cupferron'. The recovery of the iron from the cupferron precipitate was quantitatively estimated before reprecipitation as $Fe(OH)_3$, and final dissolving for electroplating as described by Peacock, Evans, Irvine, Good, Kip, Weiss & Gibson (1946). The iron was plated on to standard nickel planchets, prepared by careful cleaning with alcohol followed by nitric acid and distilled water. The cupferron stage was found to be essential to separate the iron from calcium phosphate (particularly in foetal ash specimens) which interfered with the electroplating. From the radioactive assay and the quantitative determinations, the *specific activity* of the specimen was obtained in terms of percentage of the injected dose per mg. iron. In the case of foetal and young rats, the percentage of tracer dose recovered was calculated from the relation Percentage tracer dose = Mg. iron present × *specific activity* iron.

RESULTS

The specific activity of the blood iron of control and pregnant (or lactating) rats is shown in Fig. 1. While there is considerable individual variation it is evident that the specific activity for lactating rats with litters of over 21 days is lower than the values for the controls.

The quantitative data from the ash of foetuses and young rats are summarized in Fig. 2. It will be seen that the iron content of the ash of a 21-day-old rat is three times the content at birth. The rise corresponds to about

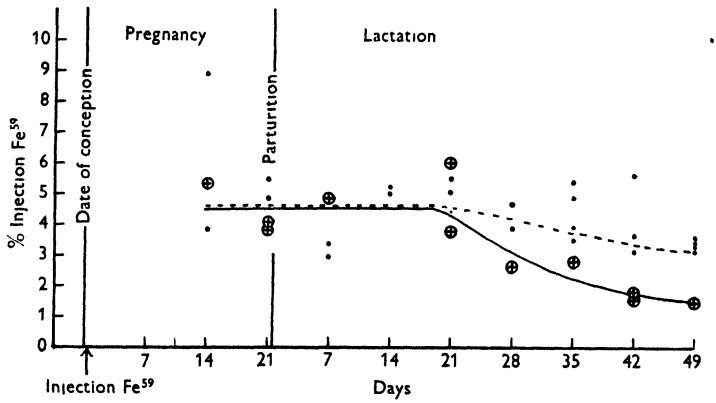

Fig. 1. 'Specific activity' of blood iron of adult rats. ⊕, Pregnant/lactating; •, non-pregnant controls.

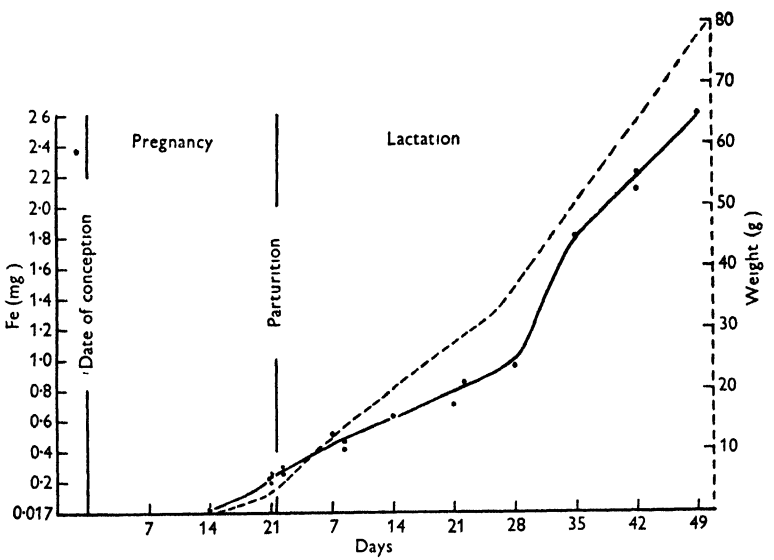

Fig. 2. Total iron and average weight in foeta and newborn rats. ——, Total iron; ----, average weight.

25 mg./kg. weight increase. The rate of the increase in iron as indicated by the full line is, however, considerably less than the rate of increase in weight for the same series of litters (dotted line).

The percentage of the injected dose of labelled iron, recovered in the whole litters killed at varying times, is shown in Fig. 3. The labelled iron recovered

from litters of two to eight rats naturally shows fairly wide variation, but the amount recovered from litters killed at 21 days or over is two or more times the labelled iron found in similar litters at birth. That the increase in iron shown in Fig. 2 comes from the lactating mother was further corroborated by transferring a litter from a non-injected rat to a recently littered injected rat as a foster mother. The young killed after 7, 14 and 21 days showed increasing amounts of radio-active iron, although clearly at birth they could have contained none.

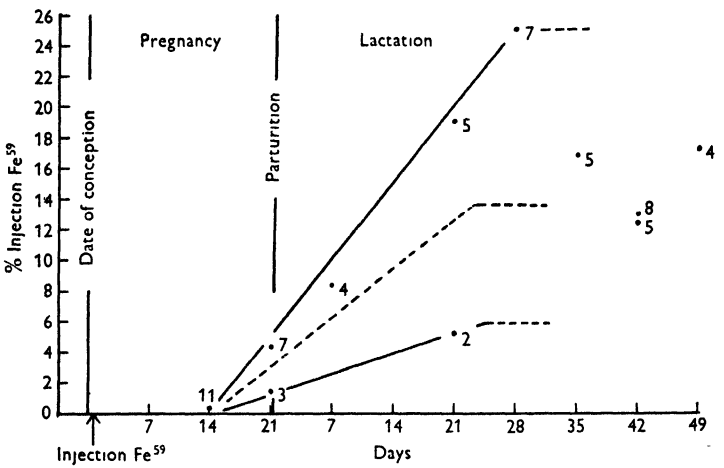

Fig. 3. Percentage labelled iron recovered from litters. ••, percentage recovered at dates indicated. Continuous lines signify upper and lower limits of recovered iron. Broken line signifies approximate mean recovery curve. Numerals signify litter size at each estimation. Weaning at 21–28 days.

The quantitative data obtained from a litter born of an injected rat but brought up by a non-injected foster mother are given in Table 1. The total iron and the ratio iron/weight fit in well with the other quantitative data obtained,

TABLE 1. Iron content of litter from mother injected with Fe^{59} but reared by non-injected foster mother

Days post-partum	1	1	8	8	15	22
Weight (g.)	5·0	5·2	10·0	12·0	20·0	31·4
Total Fe (mg.)	0·26	0·31	0·41	0·47	0·62	0·85
Fe/wt. ratio (mg./kg.)	53	59	41	39	31	27
Specific activity (% inj./mg.)	4·6	4·3	3·6	2·9	2·1	1·36
Total activity (% inj./rat)	1·21	1·32	1·49	1·17	1·33	1·16

and by calculating from the specific activity the total labelled iron in each rat (i.e. which must have been present at birth) there is good agreement throughout the litter, although individuals were of very different age and weight at the time of killing. This experiment thus provided a cross-check between the quantitative and radioactive results, and indicates that serious errors through,

for example, loss of iron in ashing, have not been incurred. It also suggests that during the first 21 days of post-natal life there is no significant excretion of the iron present at birth.

DISCUSSION

Following the experiments by Bunge between 1889 and 1892 the view has generally been held that the newborn animal was provided with an iron store which is drawn upon during the period of lactation. Although Fullerton (1937), and other workers, have drawn attention to the iron content of milk, it has been regarded as being very meagre and the pre-natal store treated as the important factor. A review of early work was given by Mackay & Goodfellow (1931).

Opinions as to the store vary. Bunge's original work was based on the high ratio of iron to weight at birth and its fall during suckling. He showed a marked fall in the ratio iron/weight in livers of newborn dogs, cats and

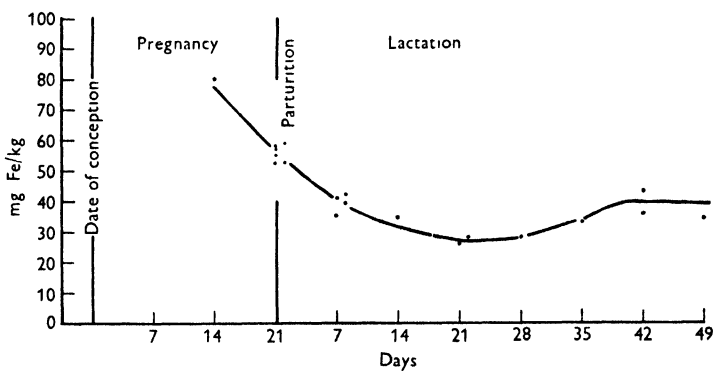

Fig. 4. Iron/weight ratio in foetal and newborn rats.

rabbits. Fullerton, reviewing the situation in human infants, emphasized the significance of iron liberated in the haemolysis which occurs in early post-natal life, and postulated that this iron was retained by the body and stored in the liver.

The experiments described above show that in rats the iron content at birth is increased threefold during the normal suckling period or, in other words, the lactating rat provides twice as much iron to its young in the 21 days following birth as in the 21 days preceding it. Nevertheless, it was pointed out that the iron increase does not keep pace with the growth rate. The data from the same series in terms of iron/weight ratios are given in Fig. 4 and demonstrate clearly the fall in this ratio to a minimum toward the end of the lactation period; after which there is some recovery due, presumably, to absorption of iron from solid diet. The high ratio iron/weight at birth may well be a fortuitous circumstance related to such possible factors in the growing foetus as the high ratio of blood volume to weight and the relative polycythaemia. Only on some basis such as

this can the even greater iron/weight ratio at the 14th day of pregnancy be accounted for. Recent work by Widdowson & McCance (1948) shows that oestrogens increase iron accumulation in adult rats, but there is no evidence that maternal oestrogens can influence iron accumulation in the foetus. In any case, the concept that the high iron/weight ratio connotes a store of iron to tide the young rat through the lactation period is totally inadequate, and this supports the findings of Venn, McCance & Widdowson (1947) in the case of piglets. The cause of the serious fall of the ratio during lactation is primarily the rapid rate of growth—the young rat grows to six times its birth weight in the same time as its iron content has increased to three times the birth value. The iron/weight ratio thus drops to half. It is this disproportion between the growth-sustaining and iron-supplying properties of milk which would ultimately lead to a state of relative iron deficiency.

The maternal commitment of iron to the litter via the milk is actually greater (for the rat) than its commitment during pregnancy, and this is reflected in the greater disparity between the specific activity of the blood iron of maternal rats which have been lactating for 21 days or more (Fig. 1) and the specific activity of the blood iron of the non-pregnant controls.

SUMMARY

1. Tracer experiments using Fe^{59} to study the iron supply of foetal and new-born rats are described.

2. There is no evidence of storage of iron at term as a special mechanism to tide the young through the nursing period. The excess iron/weight ratio at birth is probably a fortuitous circumstance.

3. The maternal commitment of iron to the litter (via the milk) may be twice as great in the 21 days following birth as in the 21 days preceding it.

4. The very rapid growth increase relative to the total iron increase is primarily responsible for the fall in the ratio of iron to weight observed during lactation. It is a disproportion between the growth-sustaining and iron-supplying properties rather than a deficiency of iron *per se* which ultimately would lead to a state of relative iron deficiency.

5. The results of these experiments suggest that the supply of iron via the milk deserves more attention than has hitherto been given.

We would like to thank Dr S. Rowlands, lately of the Physics Department, for his co-operation in constructing and testing the necessary physical and electronic equipment used for carrying out the radioactive assay of the earlier specimens and also for much valuable advice on isotope technique. We should also like to thank Mr D. A. Tanfield and Mr P. W. Roberts of the Physics Department for providing facilities for the assay of later specimens.

We are also indebted to Dr J. F. D. Frazer for general supervision of the rat colony.

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THE EQUILIBRIUM FUNCTION OF THE OTOLITH ORGANS OF THE THORNBACK RAY (*RAJA CLAVATA*)

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(*Received* 21 *July* 1949)

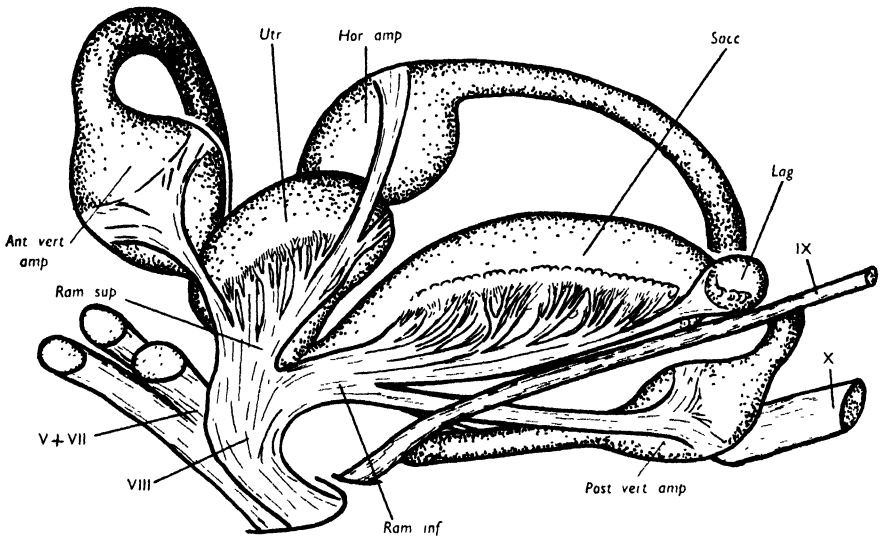
During the oscillographic study of the mode of function of the semicircular canals of the ray (Lowenstein & Sand, 1940*a*, *b*), it became clear that the accessibility of the elasmobranch labyrinth and the satisfactory survival of preparations of the labyrinth in the isolated otic capsule opened up the possibility of an extension of the work to the otolith organs in the utriculus, sacculus and lagena. This has now been carried out in experiments described in the present paper.

METHODS

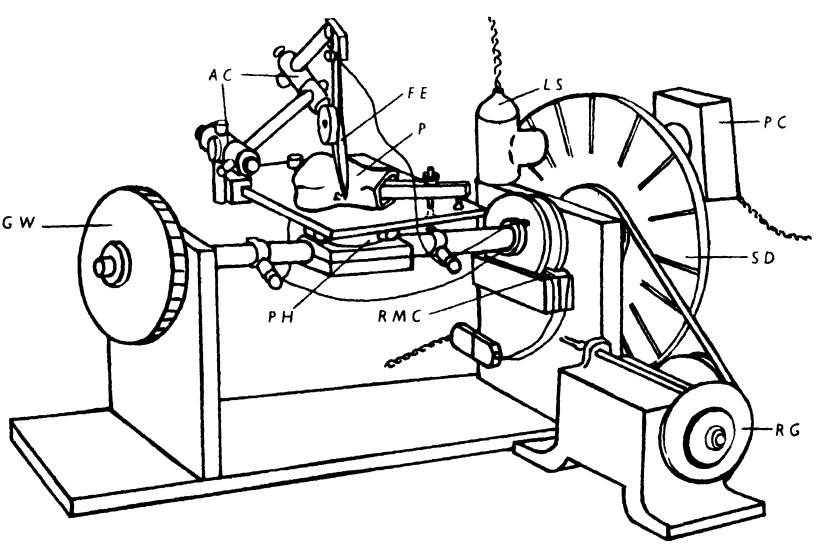
Male and female rays of a wing span of 15–24 in. were killed by decapitation, pithing, and removal of the brain. The cranium was then isolated and the labyrinth approached through the cartilage at the back of the orbit or from the ventral side. Text-fig. 1 shows a ventro-lateral view of the membranous labyrinth after a maximum exposure of the otolith organs. Openings into the perilymphatic space of a much smaller extent were made for the exposure of individual receptors. In the case of the utriculus the approach was made from the orbit, and nerve twigs from the utricular nerve fan were cut at its proximal end and peeled up towards the lateral margin of the macula. The ampullary nerve branches supplying the anterior vertical and horizontal canals were divided in order to avoid the intrusion of spurious ampullary discharges.

In the case of the ramus inferior the posterior ampullary nerve is well out of the way, and the sacculus can thus be isolated by transection of the free-running branch supplying the lagena. The latter is fortunately long enough to allow isolated pure lagena twigs to be peeled off for electrical pick-up. A choice of the region of the sacculus macula to be recorded from was possible owing to the convenient fanning out of the saccular branches of the ramus inferior. Very small twigs could be isolated which, as a rule, contained only a few functional units, the number of which could be further reduced, often to a single functional fibre, by careful crushing of the twig or by its further subdivision by splitting.

When the desired twig had been prepared it was picked up in a silver-plated forceps electrode, held in position by a number of rods connected to the preparation holder by rotary clamps. The second electrode was applied to some inner part of the cranium. The holder was then clamped to a tilting device (Text-fig. 2) with the 'nose' pointing in any desired direction. The apparatus was designed for tilting movements about any horizontal axis desired, smoothness of movement and constancy of speed being assured by the use of a 50:1 reduction gear operated by hand. The wheel at the left was graduated into 360 degrees for the accurate reading of the tilting angle. The slotted



Text-fig. 1. Ventro-lateral view of the membranous labyrinth of the ray. *Ant. vert. amp.* = anterior vertical ampulla; *Hor. amp.* = horizontal ampulla; *Lag.* = lagena; *Post. vert. amp.* = posterior vertical ampulla; *Ram. inf.* = ramus inferior; *Ram. sup.* = ramus superior; *Sacc.* = sacculus; *Utr.* = utriculus; V–X = 5th–10th cranial nerves.



Text-fig. 2. Diagram of tilting device. *A.C.* = angle clamps; *F.E.* = forceps electrode; *G.W.* = graduated wheel; *L.S.* = light source; *P.* = preparation (posterior half of cranium); *P.C.* = photoelectric cell; *P.H.* = preparation holder; *R.G.* = reduction gear; *R.M.C.* = rotary mercury contacts; *S.D.* = slotted disk.

disk at the right was made to intercept a light beam focused on a photoelectric cell which, through a relay, switched a lamp in front of the camera serving as a rotation signal for the film records. This signal gave a flash of light for every three degrees tilt and, together with a time marker (24/sec.), made possible the recording of extent, acceleration, and speed of tilting of the preparation during experiments. The preparation holder was connected with the input of a high-gain balanced resistance-capacity coupled amplifier and cathode-ray oscillograph by means of two rotary mercury contacts which worked satisfactorily without producing electrical artefacts. Apart from the cathode-ray oscillograph and the usual monitor loudspeaker, we also used an electronic impulse integrator or rate counter which enabled us to take continuous readings on a calibrated milliammeter of the rate of impulse discharge at any given moment. The advantage of this device was not only a great economy in recording film, but also the possibility of using preparations with two, three, or more active units for quantitative response analysis, film records of which would have been practically unanalysable.

Yet another recording device proved exceedingly helpful. The acoustic discharge picture from the monitor loudspeaker, readings of rotation angles, and simultaneous meter readings of the discharge frequency from the preparation were phonographically recorded by means of a recorder-play-back set obtained from Government Surplus Stores. Small diameter cellulose disks with a recording time of half an hour per side were used to record complete protocols of our experiments.

RESULTS

The results here presented are drawn from 440 experiments on 149 animals. Not all these experiments were devoted to the study of gravity responses, as some preparations yielded responses to vibrational stimuli which form the subject of a separate publication. Of the recorded experiments, 40 on the utriculus, 26 on the sacculus, and 34 on the lagena yielded analysable results.

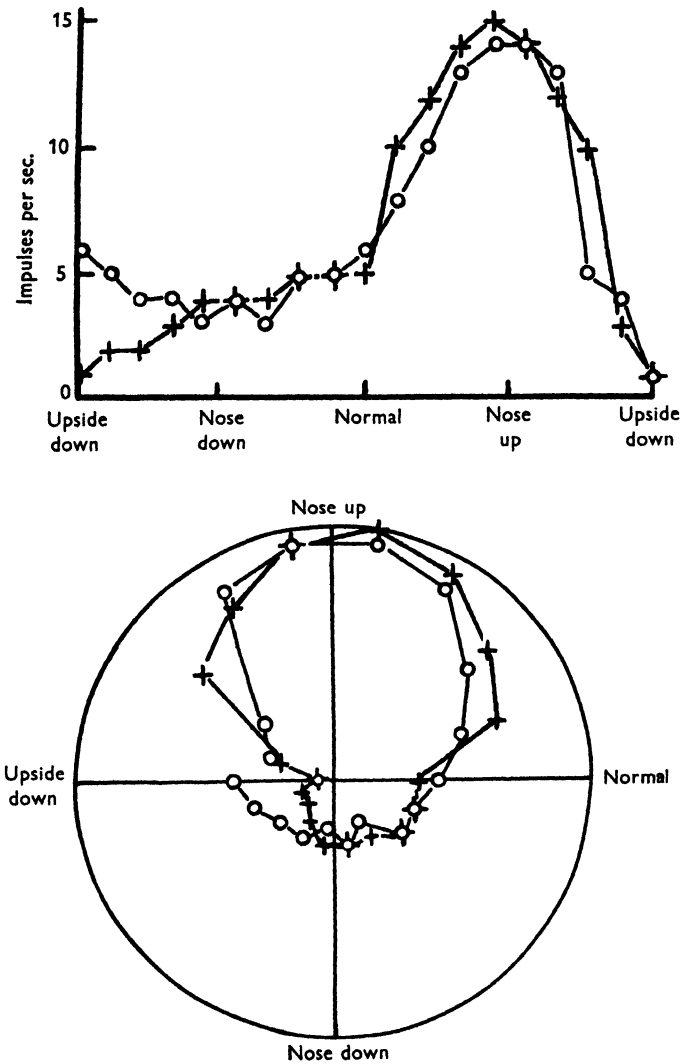
In the experiments on the response to gravity the skull was slowly tilted about a horizontal axis, the majority of the tilts being about the transverse or the longitudinal axes. The fore-and-aft tilts will be spoken of as 'towards Nose-up' or 'towards Nose-down' respectively, while the lateral tilts will be spoken of as 'Side-up' or 'Side-down'.

Utriculus

In most of the successful preparations a steady impulse discharge was observed when the nerve twig was connected to the amplifier. This steady discharge appears to be characteristic of labyrinthine sense endings (Lowenstein & Sand, 1940*a*, *b*).

Example 1 (Text-fig. 3). Connexion to the amplifier was made in this case with the skull in the Upside-down position and a steady discharge at 6 impulses/sec. (20° C.) was picked up from a single fibre. The skull was tilted slowly and steadily towards the Nose-down position: the frequency of the discharge fell. The tilting was continued at constant speed and the discharge frequency reached a minimum near the Nose-down position, rising as the Normal position was approached. The rise was steepest within 20° of Normal becoming less steep near the Nose-up position in which the frequency of discharge reached a maximum. Tilting was continued through the Upside-down position to complete a second circle without a pause. Each full circle

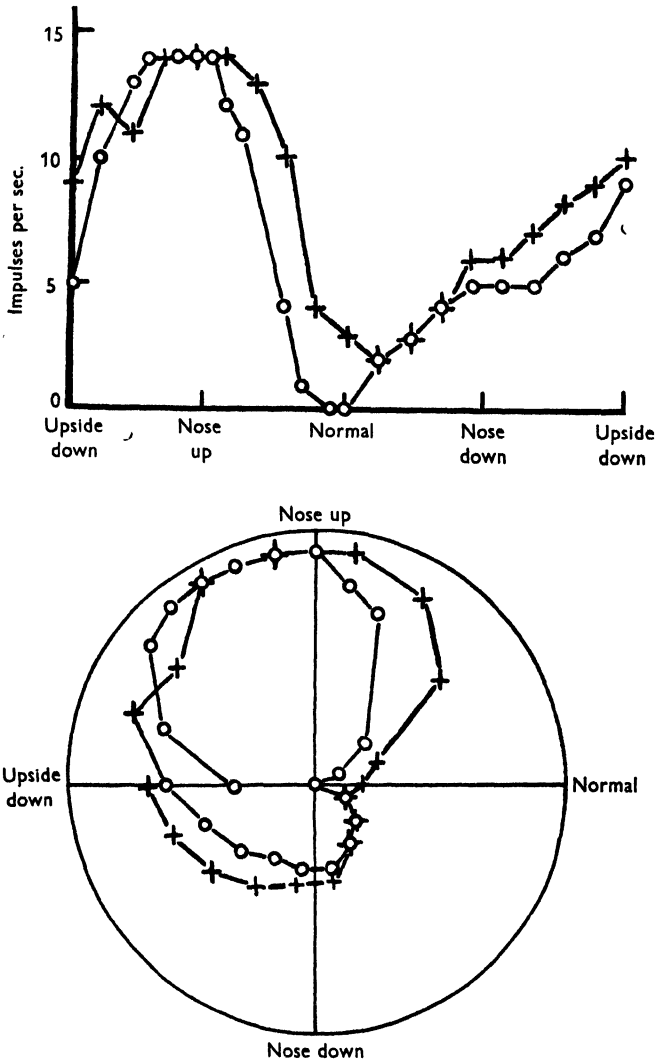
occupied 2½ min. (2·4°/sec.). The discharge frequency fell as the Upside-down position was approached, being as low as 1/sec. on passing through that



Text.-fig. 3. (Prep. 241.) Utriculus. Cartesian and polar graphs of the discharge frequencies during two consecutive full-circle fore-and-aft tilts. Both curves are to be read from left to right and counter-clockwise. ○ = first circle; + = second circle.

position. The discharge frequencies during the course of the second circle closely followed the frequencies found in the corresponding positions during the first circle. The skull was brought to rest in the Upside-down position after

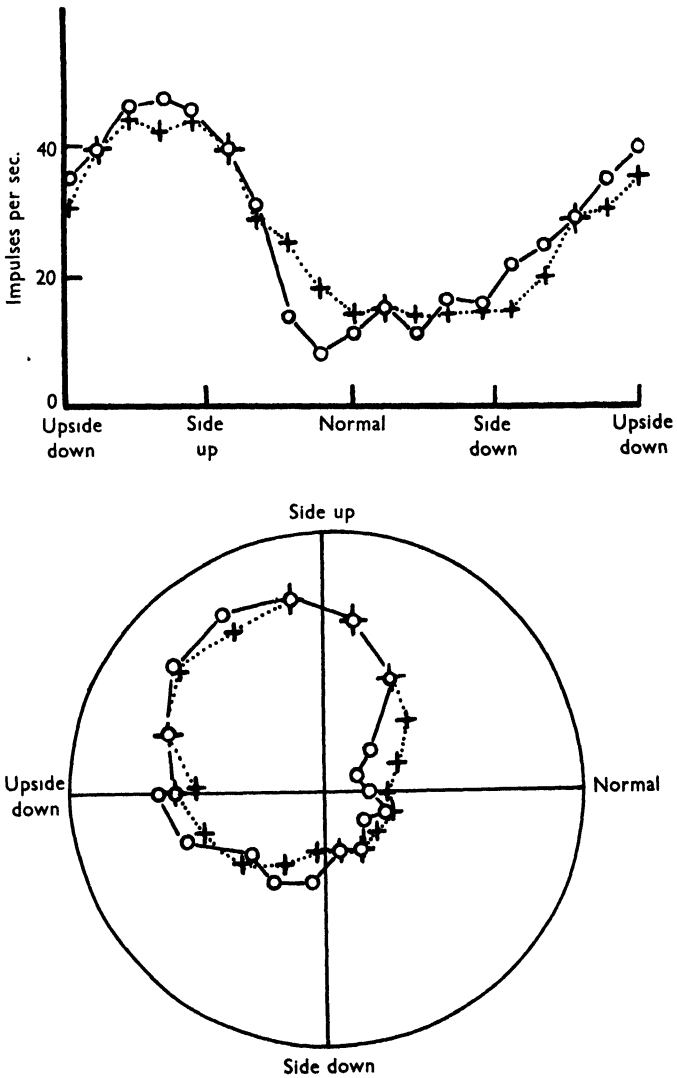
the second circle and the discharge frequency rose within a few seconds to regain the steady value of 5/sec.



Text-fig. 4. (Prep. 241.) Utriculus. As Text-fig. 3. Both curves are to be read from left to right and clockwise. ○ = first circle; + = second circle.

A tilt was now carried out in the opposite direction, towards Nose-up, through two circles at the same constant speed without a pause (Text-fig. 4). The discharge frequency rose to a maximum at Nose-up, fell off towards Normal to a minimum between Normal and Nose-down, rising again towards

10/sec. in the Upside-down position. The frequencies found during a second circle agreed closely with the frequencies in the corresponding positions during

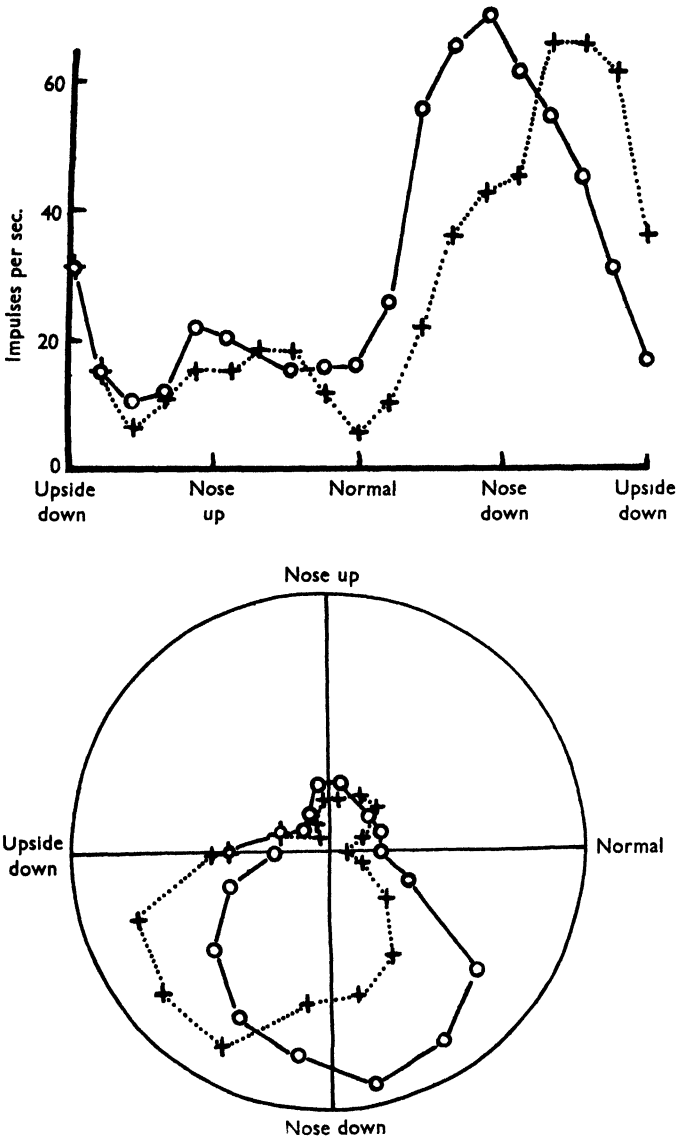


Text-fig. 5. (Prep. 286 B.) Utriculus. The discharge frequencies during two full-circle lateral tilts in opposite directions. The continuous curves to be read from left to right and clockwise, the dotted curves from right to left and counter-clockwise.

the first circle. The agreement between the circles for the two opposite directions of turning was not quite so good.

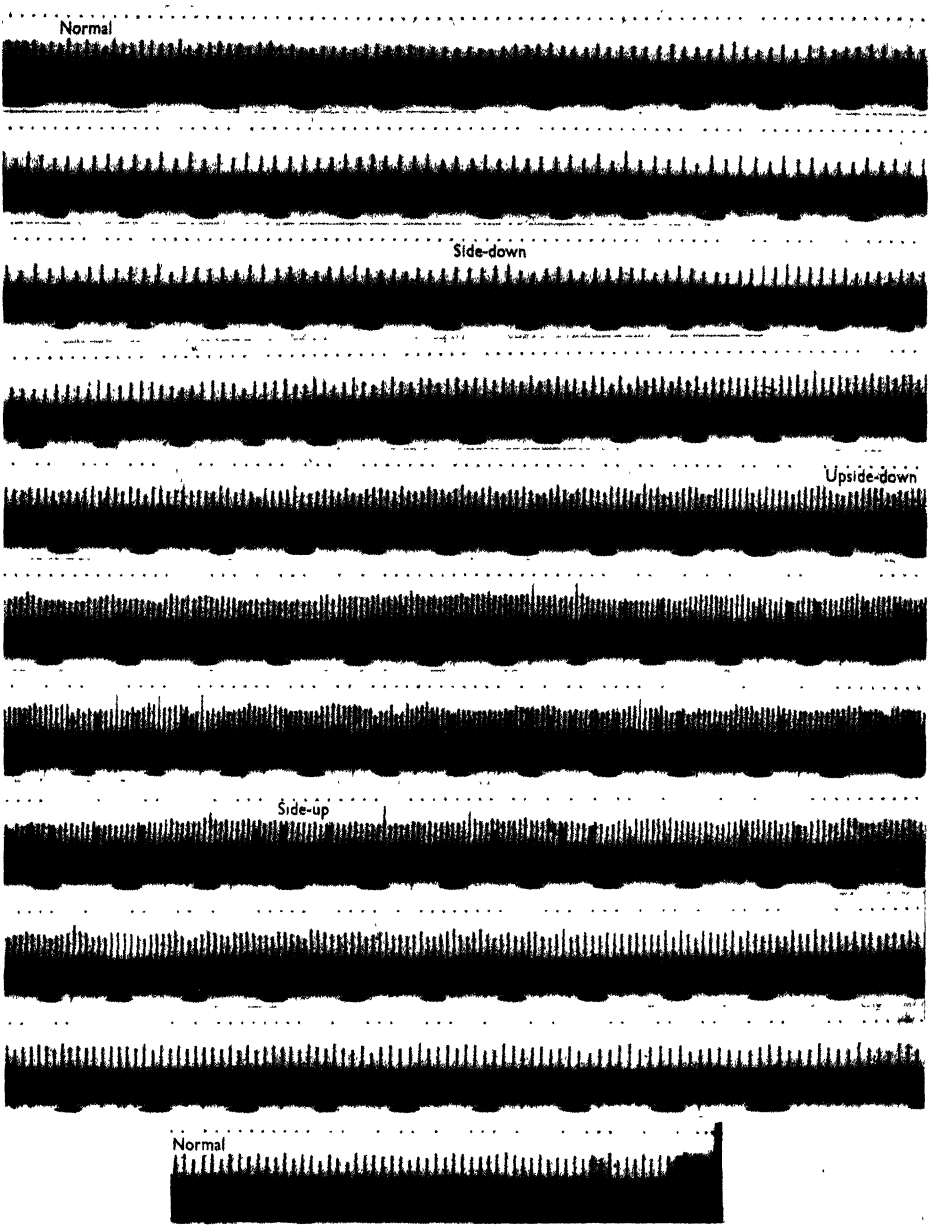
Shortly after the skull had been brought to rest in the Upside-down position the discharge frequency fell to the value of 6/sec. characteristic of this position.

The skull was now tilted into the Nose-up position and held there. The discharge frequency rose to 16/sec. Within 30 sec. of the cessation of movement



Text-fig. 6. (Prep. 286 B.) Utriculus. The discharge frequencies during two full-circle fore-and-aft tilts in opposite directions. The continuous curve to be read from left to right and clockwise, the dotted curve from right to left and counter-clockwise.

in the Nose-up position the frequency had fallen to 11/sec., and it remained very close to this value for 20 min. At the end of this period the skull was



(Prep. 288.) Utriculus: continuous film record of the response of a two-fibre preparation to a full-circle lateral tilt from Normal to Side-down to Upside-down to Side-up to Normal. Time marker at the top of record: 24/sec. Rotation signal at the bottom: one gap every 3°. Speed of rotation: 10°/sec. approx. The maximum discharge frequency lies near the Side-up and the minimum near the Side-down position.

To face p. 399

returned to the Upside-down position, whereupon the discharge frequency returned to 6/sec.

A series of 360° tilts at different speeds was now performed. There was no significant variation of the response with the speed of tilting within the range 1·5°/sec. to 5·2°/sec. On lateral tilting this particular preparation did not show any significant alteration of discharge frequency. In this respect it is by no means representative. The majority of cases, including several preparations with only a single functional unit, showed a response both on fore-and-aft and on lateral tilting.

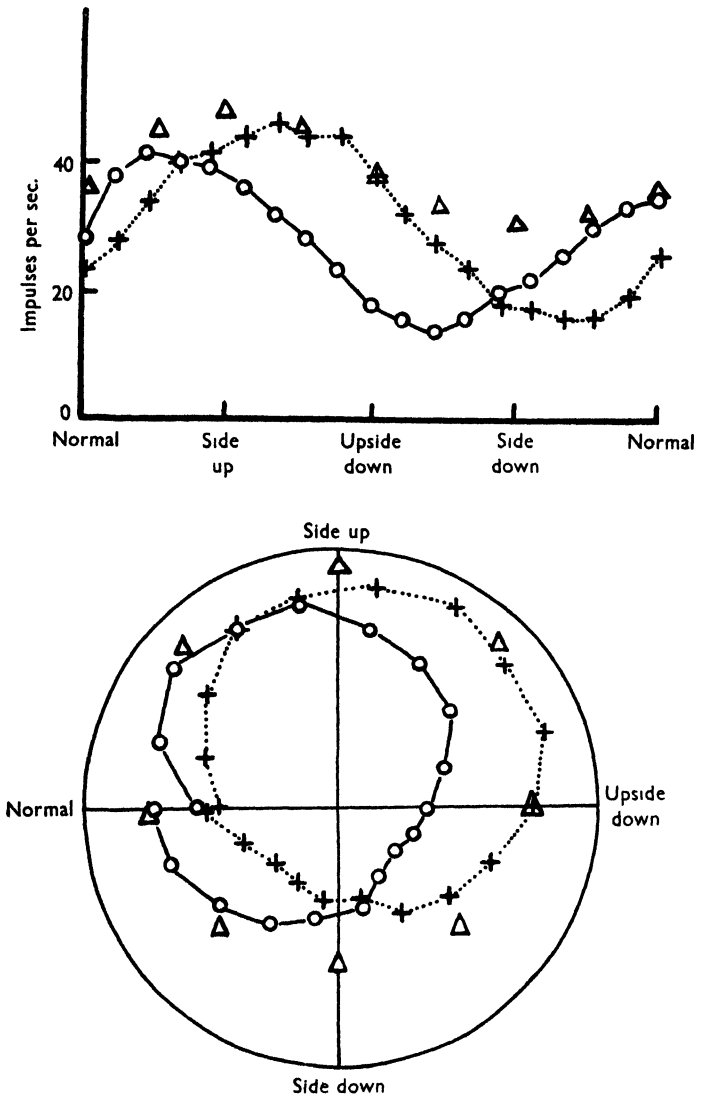
Example 2. Text-fig. 5 shows the response to lateral tilting of a 'few-fibre' preparation with a close agreement between the frequencies found in opposite directions of tilting. Text-fig. 6 shows the response of the same preparation to fore-and-aft tilting. It appears that the units contributing to the response in this case are of two types. One shows a maximum discharge frequency in a position near Nose-up, this position being independent of the direction of turning. The other shows its maximum near Nose-down, but the position at which this maximum is attained depends on the direction of tilting.

A similar dependence of the response on the direction of tilting was found in a large proportion of the preparations. The divergence between the maxima varied in extent. In some cases it was a few degrees only, whilst in others the maxima lay in opposite quadrants (Text-fig. 8).

Example 3. This preparation showed a moderate divergence in the position of the maximum both on lateral tilting and on fore-and-aft tilting. It was a two-fibre preparation in which one unit was preponderant and lent itself to separate quantitative analysis (Pl. 1). It should be noted that the unit having the smaller amplitude was inactive over a considerable part of the full circle. Apart from this the spatial characteristics of its response are similar to those of the predominant unit. Tests were also carried out with the preparation stationary in a series of positions 45° apart all round the fore-and-aft and lateral circles. The change from one position to the next was carried out very slowly, and readings of the discharge frequency were taken after the preparation had been at rest for 30 sec. Text-fig. 7 shows the relation between the steady frequencies in the various positions and the frequencies attained during continuous smooth tilts in two directions. A precisely analogous result was obtained on fore-and-aft tilting.

Example 4. In this preparation the divergence between the position of the maxima on opposite directions of tilting is as great as has been found in any preparation (Text-fig. 8). In the experiment illustrated by this figure, tilting started from the Normal position. Another experiment on the same preparation, starting in the Upside-down position, gave very similar figures. Lateral tilts were also performed starting both from Normal and Upside-down, and we were satisfied that the initial position has no significant effect on the character of

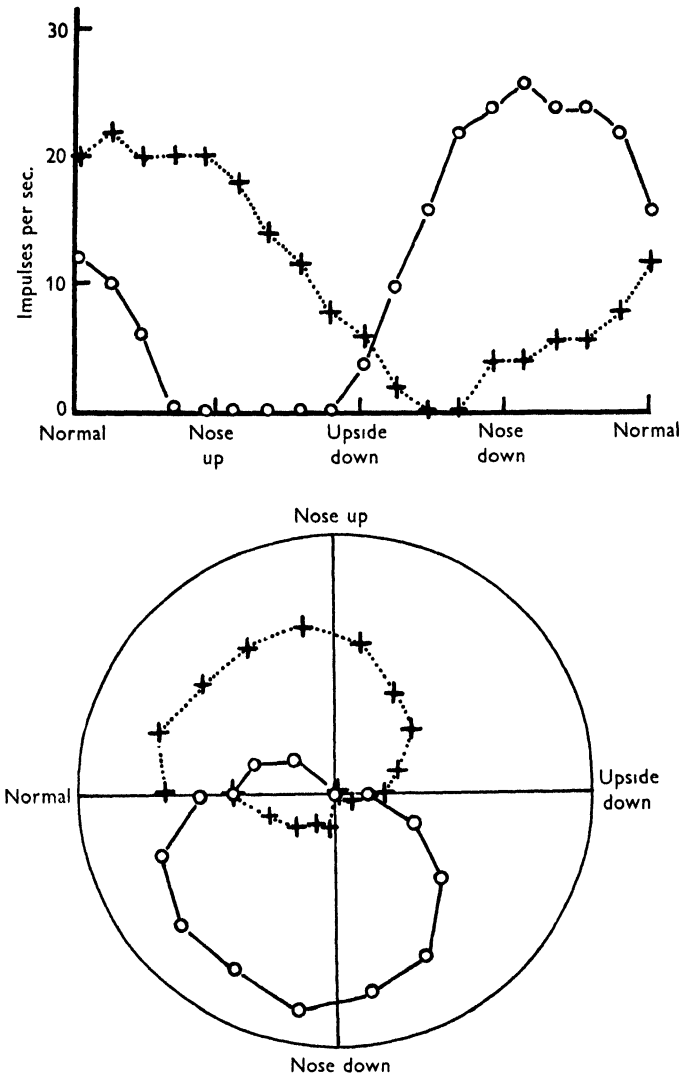
the full circle response, which depends, in this case, only on the direction of tilting.



Text-fig. 7. (Prep. 288.) Utriculus. The discharge frequencies of two full-circle lateral tilts in opposite directions. The continuous curve to be read from left to right and clockwise, the dotted curve from right to left and counter-clockwise. The superimposed frequency readings marked △ were taken during an interrupted full-circle tilt after 30 sec. rest in each position.

With this preparation also tests were made of the relation between the frequencies found during continuous turns and those found in various positions after the preparation had been held stationary. A very slow rotation was

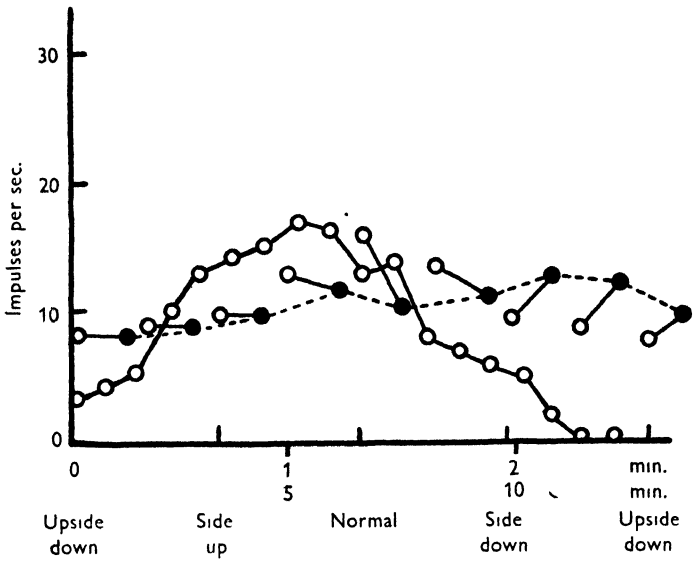
carried out (45° in 30 sec.), interrupted every 45° by a stationary period lasting 1 min. Text-fig. 9 shows the frequency readings at the beginning and at the



Text-fig. 8. (Prep. 249.) Utriculus. The discharge frequencies during two full-circle fore-and-aft tilts in opposite directions. The continuous curve to be read from left to right and clockwise, the dotted curve from right to left and counter-clockwise.

end of the stationary periods. For comparison the plottings are superimposed upon a curve of the frequency trends during a more rapid uninterrupted full circle tilt. It is clear that the discharge frequency returns in every case to a basic level and that it therefore is unsuitable to furnish a position signal. In

a preparation of this type it was also interesting to see the response to tilting about axes intermediate between the longitudinal and transverse axes. Tests about the two axes at 45° from the longitudinal gave results similar in type to those illustrated in Text-fig. 8 but with a smaller divergence between the maxima.



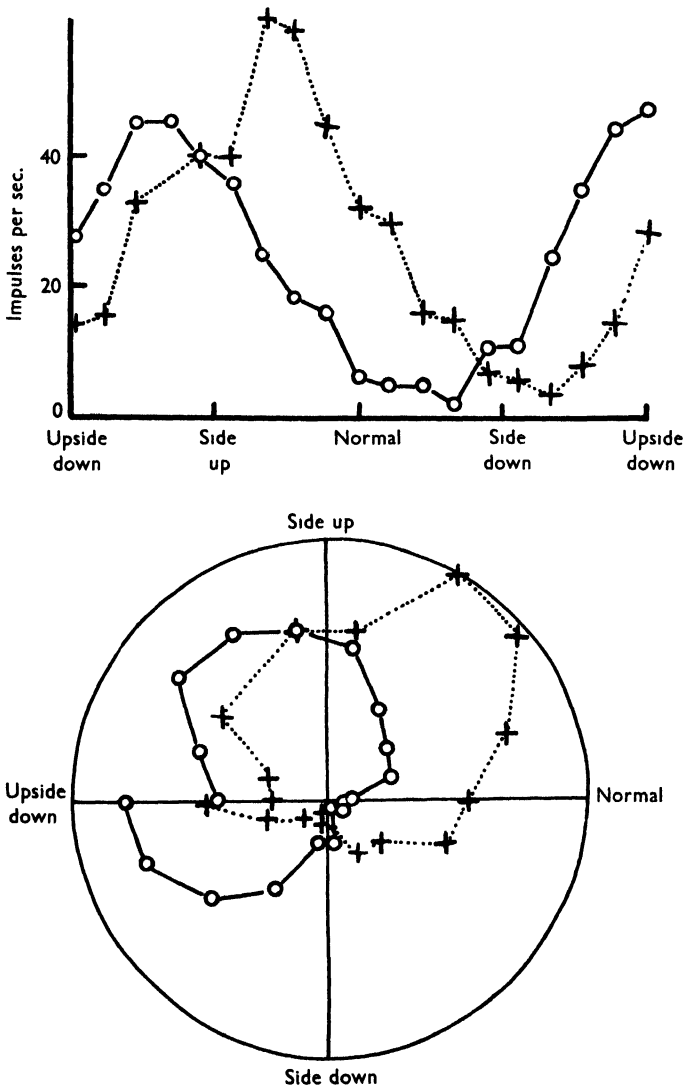
Text-fig. 9. (Prep. 249.) Utriculus. The discharge frequencies during a full-circle lateral tilt, to be read from left to right. Superimposed are the frequency readings during an interrupted tilt in the same direction. ○ = frequency on reaching position, ● = frequency after 1 min. rest in position. The latter readings are connected by a dotted line to show 'basic' discharge level.

Sacculus

In the light of the results of previous workers (Lowenstein, 1936) it would not have been surprising to find the sacculus devoid of gravity responses. This expectation has been fulfilled for a considerable area of the sacculus macula, preparations of which showed clear responses to vibrations but were not influenced by positional changes. Results gained from preparations of this type will be dealt with in a separate publication.

In each preparation in which a rotation response could be observed it took the form of a modification of a resting discharge as described for the utriculus. Despite certain difficulties which arise in the localization of a particular end-organ within a given macula (to be discussed later), it is clear that in the anterior two-thirds of the macula of the sacculus vibration receptors preponderate over gravity receptors, whereas in the posterior region the reverse is the case. The character of the responses is in some cases very similar to that found in the utriculus.

Example 5. Text-fig. 10 shows the response to lateral tilting in two directions of a 2- to 3-fibre preparation. During a second circle in each direction the



Text-fig. 10. (Prep. 269A.) Sacculus. The discharge frequencies during two full-circle lateral tilts in opposite directions. The continuous curves to be read from left to right and clockwise, the dotted curves from right to left and counter-clockwise.

sequence of discharge rates (not shown in the figure) closely followed that found during the first circle. It will be seen that the response in this case resembles the utricular response shown in Text-fig. 7.

Example 6. In another preparation lateral tilting produced a response of a type essentially similar to that illustrated by Text-fig. 5 for the utriculus.

The results from the sacculus thus display a range of response types similar to that described for the utriculus. Preparations showing a maximum discharge frequency in or near the Nose-down position appeared to predominate in the sacculus, whereas in the utriculus maxima in or near the Nose-up position were more frequently encountered. Preparations showing maxima near the Side-down position were equally rare in both organs.

In preparations of the type so far described the change in discharge frequency is continuous throughout the full-circle rotation. The response of some receptors, however, is limited to a part only of the full circle, the discharge frequency remaining steady during the rest of the circle, sometimes for more than 180° of tilting. In such cases the response may appear as a series of 'bursts', each persisting for only a short range of tilting (10–40°). The bursts do not occur at the same positions in successive circles, and for this reason we have come to the conclusion that these latter preparations may not be representative of the natural behaviour of the sense-organ.

The sacculus does not differ materially from the utriculus in its capacity for signalling maintained displacements by characteristic discharge frequencies.

Lagena

The most striking features of the behaviour of the lagena are the uniformity of the response patterns obtained and the complete freedom from vibration sensitivity.

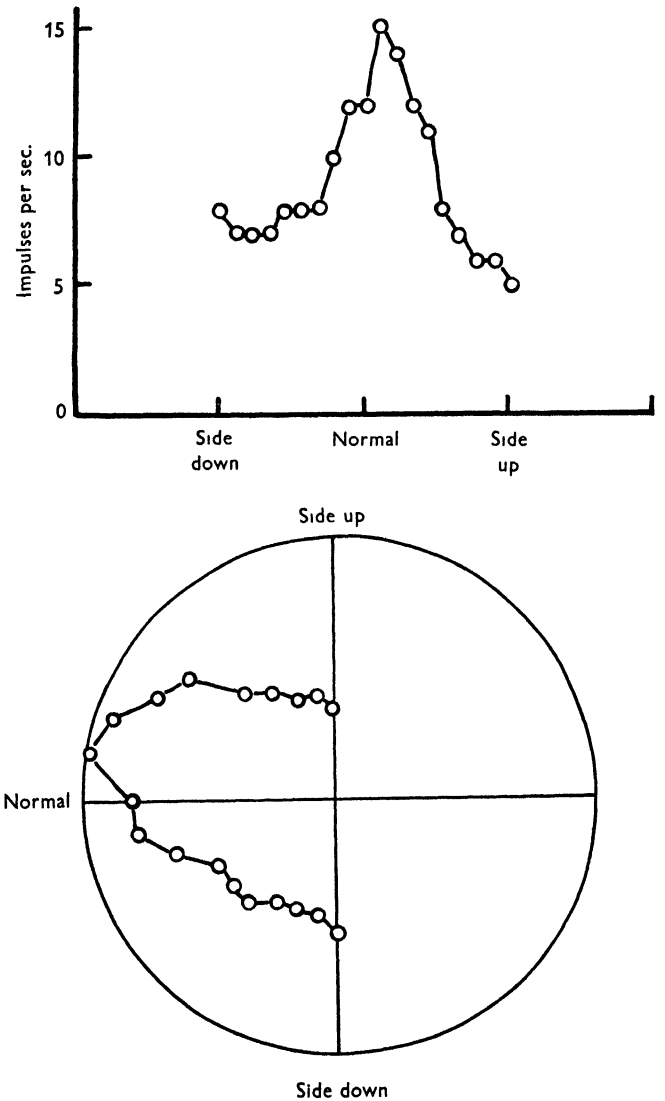
Example 7. Text-fig. 11 shows the response of a single functional unit during a lateral tilt from Side-down to Side-up through Normal. It will be seen that the maximum is comparatively sharp and that it occurs near the normal position.

This behaviour is typical of most of the preparations observed, and the deviations of the maximum from the Normal position in these cases are generally less than 30°. The discharge frequencies in various positions are, in these typical cases, independent of the direction of tilting. A similar discharge pattern is obtained on fore-and-aft tilting; here, too, the discharge frequency rises steeply as the normal position is approached from either direction.

In some preparations with more than one functional unit, a second (usually smaller maximum occurred near the Upside-down position (Text-fig. 13). We have no evidence as to whether such a second maximum can occur in the response of a single unit.

A considerable number of sense endings in the lagena are silent for more than 180° of tilting, becoming active in rapid succession on approaching the Normal position. In consequence, a preparation which in the Upside-down position has the appearance of containing only a single unit may, in the Normal

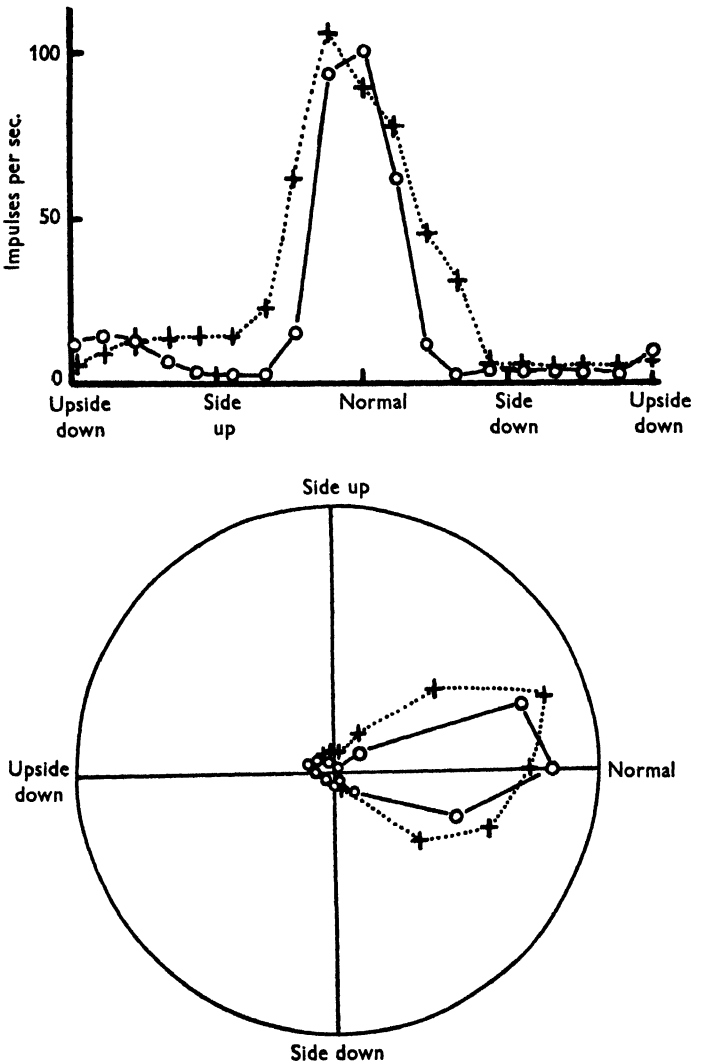
position, show a massive discharge of very high frequency. *Example* 8 (Text-fig. 12) shows such a response picture. Some preparations have been obtained



Text-fig. 11. (Prep. 129.) Lagena. The discharge frequencies during a 180° lateral tilt. To be read from left to right and clockwise.

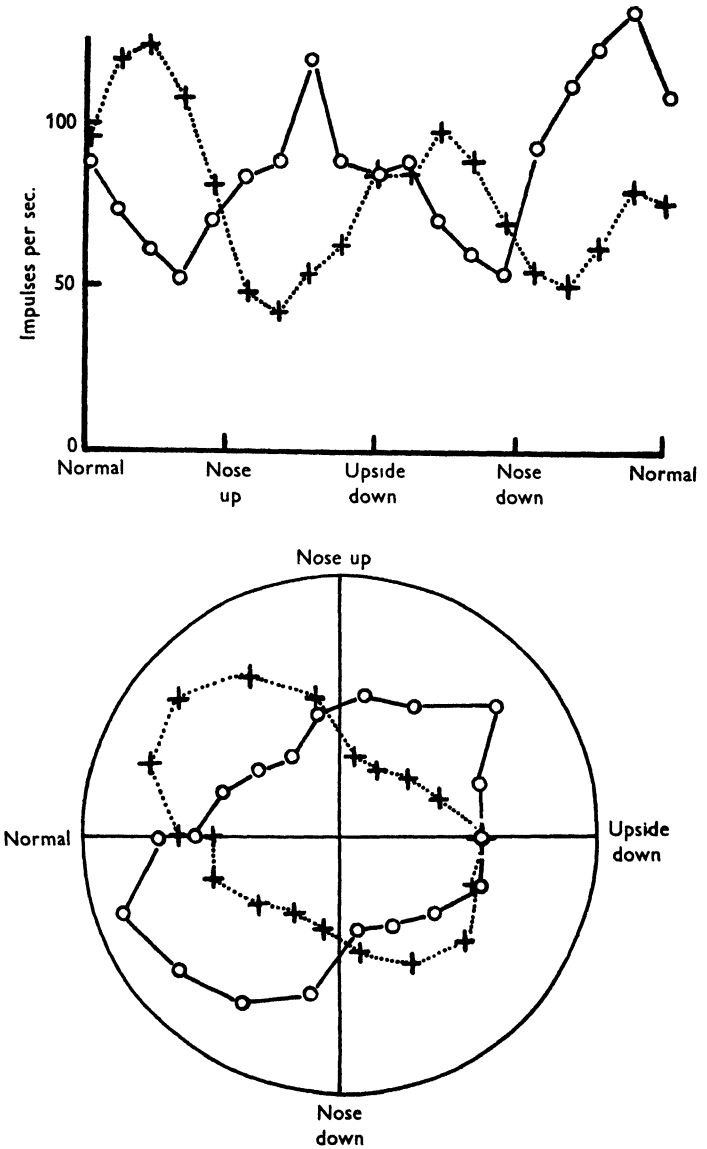
in which the maximum discharge frequency occurs in different positions according to the direction of tilting. *Example* 9 (Text-fig. 13) shows such a response from a few-fibre preparation to fore-and-aft tilting in two directions.

Example 10 (Text-fig. 14) shows the relation between the discharge frequencies in various positions during a smooth tilt and those found while the preparation



Text-fig. 12. (Prep. 213.) Lagena. The discharge frequencies during two full-circle lateral tilts. The continuous curves to be read from left to right and clockwise, the dotted curves from right to left and counter-clockwise.

was held stationary in the corresponding positions for 3 min. It is clear from this experiment that the lagena, too, contains sense endings which satisfy the requirements for positional receptors.

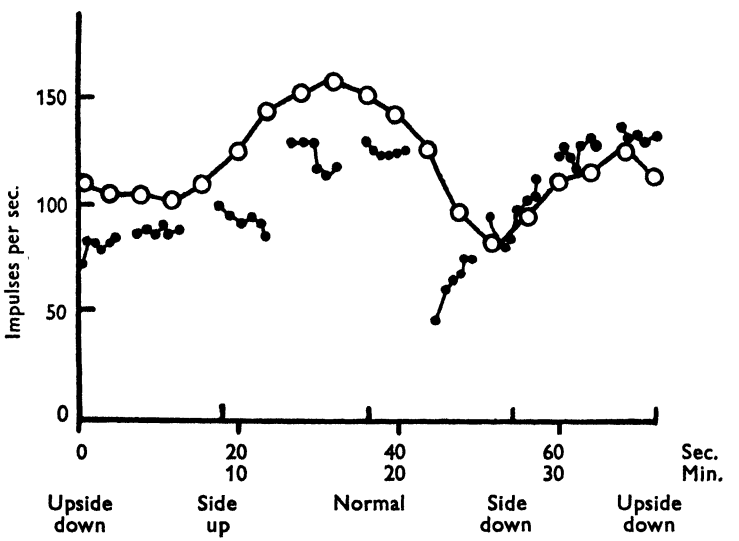


Text-fig. 13. (Prep. 76.) Lagena. The discharge frequencies during two full-circle fore-and-aft tilts. The continuous curves to be read from left to right and clockwise, the dotted curves from right to left and counter-clockwise.

Responses to linear translations

Tests were finally carried out with the object of recording the responses of the gravity receptors in the otolith organs to linear translations. As we had no provisions for the application of quantitatively controlled stimuli of this kind

the tests were improvised and qualitative only. The preparation holder held in the hand of the operator was moved forwards and backwards, from side to side, and up and down. The absence of signals made it impossible to subject the photographic records to an accurate analysis. It was, however, clear that increases and decreases in the discharge frequency occurred on opposite movements, and that an increased discharge appeared to be correlated with inertia movements of the otolith mass away from the macula and vice versa. For technical reasons no tests could be carried out for responses to centrifugal force. The existence of such reactions must, however, be postulated in organs designed to respond to linear accelerations.



Text-fig. 14. (Prep. 227.) Lagena. The discharge frequencies during a full-circle lateral tilt, to be read from left to right. ○ = frequency readings during continuous tilt (time scale, sec.), ● = frequency readings with preparation at rest in different positions (time scale min.).

DISCUSSION

In the labyrinth of the ray all three otolith organs, viz. utriculus, sacculus, and lagena participate in the maintenance of equilibrium. This result, although in full agreement with the classical conception of labyrinth function, may be considered surprising in the light of relatively recent work on a number of different vertebrate types ranging from bony fishes to mammals, where elimination of the sacculus or the sacculus-lagena complex did not significantly affect any of the known labyrinthine reflexes (Lowenstein, 1936). Considering the phylogenetic position of the Elasmobranchs, however, it is highly interesting to find in a member of this group the generalized functional picture represented by our results. Breuer (1891) postulated in his classical theory of otolith function a division of labour between the otolith organs in which their range of

response, like that of the semicircular canals, was strictly correlated with their anatomical arrangement with respect to the three main planes of space. Our results do not bear this out. There is a wide overlap in functional range between the otolith organs, as their sense-endings were generally found to respond to tilts about both the longitudinal and the transverse axis. Adrian (1943) reports a similar behaviour of some of the gravity-controlled units in the brain stem of the cat. The lagena does show a typical response picture of its own, but this bears no relation to Breuer's speculation, and owing to its absence in mammals the organ plays no part in the later functional theories put forward by Quix (1925) and Magnus & de Kleijn (1926 *a*, *b*).

It is clear from the similarity in response from the utriculus and the sacculus that, during deviation from the normal position, a considerable number of receptor units in both organs must be sending almost identical and thus mutually reinforcing signals to the central nervous system. It may, therefore, be permissible to assume that either of the two organs could phylogenetically be switched to another task, such as vibration reception, without depriving the labyrinth of any specific category of gravity reception. This appears, in fact, to have happened several times in vertebrate evolution. The bony fish represent a good example. In the Cyprinoids there is strong evidence that the sacculus and lagena complex can be eliminated without any noticeable effect on equilibration (Lowenstein, 1932). A remarkable capacity for hearing and sound discrimination was shown to be localized in this part of the labyrinth (v. Frisch, 1936). In the Clupeids there are strong indications that the acoustic function of the ear may have its main seat in the utriculus, and the possibility cannot be ruled out that here the sacculus may be the chief equilibrium organ among the otolith-bearing structures of the labyrinth (de Burlet, 1935).

The vibration sensitive sense-endings in the elasmobranch labyrinth are found in the utriculus (lacinia), in a large part of the sacculus macula, and in the macula neglecta. These results will be presented in a separate paper in this *Journal*. The macula lagenae is wholly made up of gravity receptors. The phylogenetic fate of this sense-ending is interesting. In some bony fish it is probably concerned with hearing. In Amphibia its loss was claimed by McNally & Tait (1925) to be without effect on equilibrium, but it has now been shown by MacNaughton & McNally (1946) that the unilateral loss does bring about a deficiency pose of the head consisting of slight homolateral upward tilts, whereas the only consequence of bilateral elimination of the lagena is oscillation of the head on coming to rest after spontaneous movement, which amounts to a deficient stabilization of the head near the normal position. Possession of the lagenae after elimination of all other labyrinthine sense-endings preserves a prompt righting reflex absent in animals after total bilateral labyrinth elimination. These findings in the Amphibia agree well with the responses recorded from the elasmobranch labyrinth, which show the

lagena as a marker for the normal position. It is tempting to speculate, that the gravity responses recorded by Ross (1936) from the ramus inferior of the eighth nerve of the frog may have been derived from the lagena. In birds the lagena becomes separated from the sacculus by its close association with the tip of the cochlear outgrowth, and in mammals it is missing altogether. A gradual loss of equilibrium function and, probably, association with sound perception precedes its final disappearance. It is thus quite clear that the otolith organs show great functional plasticity, their potentialities being foreshadowed in the generalized conditions obtaining in the elasmobranch labyrinth.

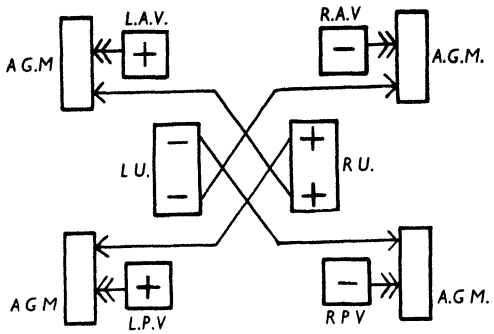
A question of great interest in the study of a 'stato-receptor' is whether it is capable of signalling persistent deviations from the normal spatial orientation. Theoretically one would expect such a receptor to have a characteristic discharge rate for every position, or at least for a number of critical orientations. Sense-endings approaching this ideal type are represented by examples illustrated in Text-fig. 7 (utriculus) and Text-fig. 14 (lagena). They have also been found in the sacculus. When in such a case the preparation is very slowly tilted from Normal to a certain position in space, the frequency of discharge increases or decreases. When the preparation is then held stationary in the 'abnormal' position the increased or decreased discharge frequency reverts somewhat towards the initial level, remaining, however, significantly above or below it for a matter of minutes.

Prejudiced by the universally accepted prototype of a 'stato-receptor' we were somewhat surprised to find that a considerable proportion of the sense-endings in the three maculae did not show this capacity to signal, in this way, maintained deviations from the normal spatial position. An extreme case of this type is illustrated by Text-fig. 8 (utriculus). This receptor shows a marked fall in activity whenever the normal position is left, moving towards a minimum irrespective of whether the labyrinth is lowered or raised. After transit through the Upside-down position its activity rises towards a maximum, whatever the direction of rotation. Text-fig. 9 shows that the activity reverts to a basic level whenever the tilt is interrupted for any length of time. Such a receptor cannot by any stretch of imagination be called a 'stato-receptor'. It is, however, suited to signal positional change as such, and may be described as an out-of-position receptor. The semicircular canals fulfil a similar task. But they react to angular accelerations only, whereas the gravity-controlled out-of-position receptors respond to constant-speed spatial deviations.

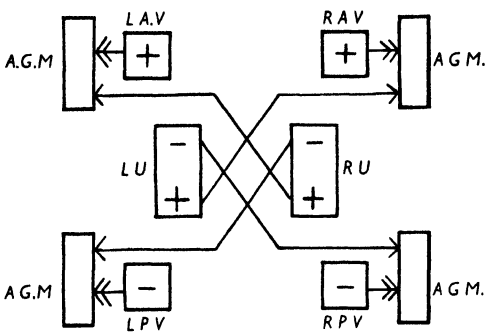
In yet another respect our results are in evident contradiction to the hitherto generally accepted model of otolith function. The effect of unilateral total labyrinthectomy in all vertebrate classes is primarily a loss of anti-gravity tonus on the operated side which leads to the well-known rolling over to this side during forward progression. As this tonus asymmetry is accentuated by

a tilt to the normal position, and is least noticeable when the animal comes to rest lying on its operated side, the conclusion was naturally reached that the otolith organ responsible for the perception of lateral tilts is maximally stimulated when the labyrinth is lowered. The findings of Tait & McNally (1934) in what they call the uni-soliutricular frogs, in which all labyrinthine receptors except one utriculus were eliminated by nerve cutting, also point towards ipsilateral preponderance of the anti-gravity muscles under the influence of the intact vestibular end organ. In agreement with this, all records obtained from the vestibular nucleus of the mammalian brain stem (Adrian, 1943) show increased impulse discharge on side-down tilting, which has been tentatively attributed to the activity of the ipsilateral sacculus. Consequently, we expected to find that at least a majority of sense-endings in the utriculus or sacculus, or both, increase their discharge frequency on side-down tilting. The opposite is the case. We are inclined to believe that the regions of the maculae from which we recorded were fairly representative of the whole; yet all sense-endings capable of signalling maintained deviations from the Normal showed a decreased activity in or near Side-down, and conversely a maximum in or near Side-up positions.

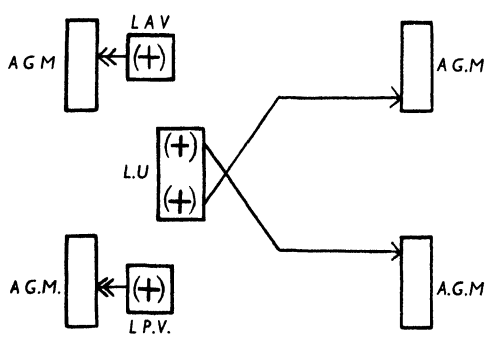
The explored parts of the utriculus and sacculus maculae contain at least two types of sense-endings, one responding with an increase in discharge to Side-up and Nose-up, the other to Side-up and Nose-down displacements. Nerve fibres from these two types can be found side by side in one and the same nerve twig. This does not, however, mean that they are necessarily derived from neighbouring neuromasts, as a considerable amount of fanning-out is characteristic of the end-ramifications of the nerve strands. It is thus difficult to localize the exact position of the sense-endings concerned with sufficient confidence to justify far-reaching theoretical deductions. From the fact, however, that the lateral parts of the utriculus macula yield preponderantly preparations whose discharge frequency increases on Side-up tilting, it may be tentatively suggested that either a gliding away of the otolith, producing a medial deflexion of the sense hairs of the neuromasts or a relief of the vertical pressure of the otolith on the macula, may represent the appropriate stimulus, with the reverse hair deformation or pressure change producing a reduction in the discharge frequency. If this were correct then the Side-up and Nose-up organs ought to be located in the antero-lateral, the Side-up and Nose-down organs in the postero-lateral part of the utriculus macula. This localization would be in full agreement with the stimulation experiments on the dogfish (Maxwell, 1923) where it was found that pressure on the otolith applied laterally, so as to move the otolith slightly inwards, produced eye responses corresponding to those occurring on the lateral up-tilting of the stimulated labyrinth. Pressure on the anterior side of the otolith produced the effect of Nose-up tilting, and pressure on the posterior end the opposite. From this



(a)



(b)



(c)

Text-fig. 15. Diagrammatic representation of the hypothetical tonus-distribution from vertical canals and gravity receptors to the anti-gravity muscles of the four limbs: (*a*) during lateral tilt to the left; (*b*) during Nose-down tilt; (*c*) in the normal position after elimination of the right labyrinth. *A.G.M.* = anti-gravity muscles; *L.A.V.* = left anterior vertical canal; *L.P.V.* = left posterior vertical canal; *L.U.* = left utricle; *R.A.V.* = right anterior vertical canal; *R.P.V.* = right posterior vertical canal; *R.U.* = right utricle. The + sign denotes an increase, the − sign a decrease in the discharge frequency during tilting movements. (+) indicates the tonus effect of the resting discharge in the stationary labyrinth. All four vertical canals would be marked (+) in the absence of angular acceleration. The double arrow-head from the canals symbolizes their assumed preponderance (see text).

Maxwell concluded that the effective stimulus is a displacement of the otolith. This satisfactory agreement based on two entirely different methods is not supported, however, by the results of similar stimulation experiments on the pike (Ulrich, 1935). Here the effects of delicately apportioned pressures on the utriculus otolith are directly opposed to those described by Maxwell.

If any permanent association between certain macula areas and groups of effector muscles existed, this would mean that the utriculus and sacculus maculae in a tetrapod are preponderantly in control of the contralateral anti-gravity muscles, with the anterior and posterior parts of the maculae controlling the posterior and anterior pairs of limbs respectively (Text-fig. 15). It will be seen from the diagrammatic representation of this hypothetical scheme that the otolith organs are assumed to be antagonistically pitted against the vertical canals spatially associated with them. Text-fig. 15*a*, *b* illustrate the distribution of excitation on a left Side-down and a Nose-down tilt, with the plus and minus signs indicating increased and decreased discharge-activities respectively. The apparent discrepancy between the deficiency phenomena found after unilateral total labyrinth elimination and our electro-physiological findings can be overcome by making the additional assumption that the sum total of the tonic influence of the semicircular canals, apart from being opposed to that of the otolith organs, is also significantly stronger. Such an assumption would lead back to Ewald's theory of the 'tonus labyrinth' in which the canals were described as the chief source of labyrinth tonus. Text-fig. 15*c* illustrates the distribution of excitation after total elimination of the right labyrinth, and it will be seen that the typical deficiency phenomena based on tonus preponderance on the intact side can be readily deduced from it. Although it is very tempting to adopt some such *ad hoc* scheme of explanation, it must be said that the pattern of functional integration is probably more complicated. A significant indication that this may be so, is the fact that Tait & McNally (1934), in their interpretation of elimination experiments on the frog's labyrinth, found themselves forced to abandon the prevailing idea that the otolith 'acts by a simple and uniform sliding descent from a higher to a lower level' and came to the conclusion that a 'hypothesis of a point-to-point independence of the elements composing the otolith organs is unavoidable'.

A central projection of the 'macula map' as a whole may be assumed to furnish the substrate for a side-by-side and moment-to-moment representation of the sum total of deformation effects in the maculae, with the effector system reacting to the stimulus situation as a whole rather than to discrete aspects of it. Such a picture would be very acceptable, if it did not by its very nature defy experimental analysis. But this is a difficulty common to a number of sensory fields.

SUMMARY

1. In the labyrinth of *Raja clavata* all three otolith organs, viz. the utriculus, sacculus, and lagena participate in the maintenance of equilibrium.

2. Sense-endings in the maculae generally show a resting discharge, the frequency of which is increased or decreased by positional changes.

3. The functional ranges of utriculus and sacculus overlap. Both contain sense-endings responding to lateral and fore-and-aft tilting. There are two main types which have their maximum of discharge activity in Side-up and Nose-up and Side-up and Nose-down positions respectively. Organs having a maximum in the Side-down position were encountered, but did not appear among the position-receptors proper.

4. Apart from 'static' position receptors, the maculae contain receptors responding to a change of position in one and the same manner, irrespective of the direction of the change. They are described as 'out-of-position' receptors.

5. The receptors in the lagena also respond both to lateral and to fore-and-aft tilts. They have their maximum of activity usually in or near the normal position and can be described as 'into-level' receptors.

6. The otolith organs show clear reactions to linear translations in the three planes of space. These, and theoretically postulated reactions to centrifugal force, have not yet been subjected to a quantitative analysis.

7. The theoretical implications of the electro-physiological findings are discussed.

The work was carried out at the Laboratory of the Marine Biological Association at Plymouth with the aid of a Royal Society grant from the Parliamentary Grant-in-aid of Scientific Investigations, while one of us (O.L.) was privileged to hold the Tables of the British Association (1947) and the Physiological Society (1948) at that Laboratory. Our special thanks are due to the Director and staff of the Laboratory for their most generous support in providing the considerable amounts of living material and technical research facilities, without which the work would have been impossible. Part of the electrical equipment was built in the Natural Philosophy Department of the University of Glasgow, and we are greatly indebted to Prof. P. I. Dee, C.B.E., F.R.S., and Dr W. M'Farlane for generously putting the facilities of the Department and workshop at our disposal.

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THE PENETRATION OF SOME SULPHONAMIDES INTO THE INTRA-OCULAR FLUIDS OF THE CAT AND RABBIT

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Earlier work from this laboratory on the barrier between blood and intra-ocular fluids has been confined to the study of such strongly water-soluble substances as sugars, urea, amino-acids and various inorganic ions. Palm (1947) has shown that ethyl alcohol penetrates into the aqueous humour at least five times as rapidly as the fastest water-soluble substance studied, so that it is of interest to extend the work to further substances in which lipoid solubility is of significance. In the present work four sulphonamides, namely sulphanilamide, sulphapyridine, sulphadiazine, and sulphathiazole, have been studied.

METHODS

General. Experiments on anaesthetized cats were carried out as described earlier (Davson & Duke-Elder, 1948), without tying the renal arteries. The sulphonamide was injected as an approximately neutral isotonic solution, the concentration in the blood being maintained approximately constant by further repeated injections. Eyes were enucleated at appropriate intervals and the aqueous humour and vitreous body removed for analysis. Experiments on rabbits were nearly all done on conscious animals with only local anaesthesia; the sulphonamide was injected from a syringe connected by a long rubber tube with a needle in the marginal ear vein; blood samples were drawn from the vein of the other ear. From the first eye, only aqueous humour was removed under topical anaesthesia; the second eye was enucleated immediately after anaesthetizing the animal with nembutal, and both aqueous humour and vitreous body were analysed.

Analytical. Sulphonamides were estimated by the method of Bratton & Marshall (1939) in trichloracetic acid filtrates. Since an appreciable part of the sulphonamide in the blood is bound to protein, it is of importance for calculating the permeability constant to estimate the concentration of sulphonamide in a saline solution that comes into diffusion equilibrium with the plasma under the conditions of the experiment. To this end blood plasma, taken at the end of the experiment, was dialysed against saline containing approximately the same concentration of sulphonamide; the dialysis chamber described earlier (Davson, Duke-Elder & Maurice, 1949), was employed.

The ratios: concentration in saline/apparent concentration in plasma are not greatly different in the two species. When mean values for this ratio were employed, i.e. when for some reason the dialysis was not carried out, the following values were used: 0·65, 0·81, 0·73, 0·53 for sulphapyridine, sulphanilamide, sulphadiazine and sulphathiazole respectively. Individual deviations of more than 10% of these values were rarely encountered.

RESULTS

The results of experiments on twenty-four cats and forty-four rabbits are summarized in Tables 1 and 2; the relative rates of penetration into the aqueous humour and vitreous body are indicated by the parameters K_A and K_V (Davson & Quilliam, 1940). This treatment is based on the assumption that, at

TABLE 1. Penetration of sulphonamides into the intra-ocular fluids of the cat

No. of exps.	Substance	Time interval (min.) 0–10 K_A	10–20 K_A	0–20 K_A	0–20 K_V
12	Sulphapyridine	91 ± 8	48 ± 4	70 ± 5	25 ± 2
7	Sulphanilamide	76 ± 9·5	53 ± 7·5	64 ± 6	23 ± 1
5	Sulphathiazole	32 ± 5·5	21 ± 3·5	26·5 ± 5	6·4 ± 2

TABLE 2. Penetration of sulphonamides into the intra-ocular fluids of the rabbit

No. of exps.	Substance	Time interval (min.) 0–10 K_A	10–20 K_A	0–20 K_A	0–20 K_V
7	Sulphapyridine	100 ± 8	48 ± 7	74 ± 7	29 ± 1
7–9	Sulphanilamide	96 ± 6	69 ± 7	83 ± 5·5	31 ± 1
7–13	Sulphadiazine	50·5 ± 3	27·5 ± 4·5	42 ± 3	9·7 ± 0·5
9–15	Sulphathiazole	32 ± 2·5	15 ± 2	24 ± 3	5·5 ± 0·4

K_A is given by $\frac{100}{t} \log \frac{C_{Pl} - C_{Aq_1}}{C_{Pl} - C_{Aq_2}}$, where C_{Pl} is the concentration in the plasma and C_{Aq_1} and C_{Aq_2} are the concentrations in the aqueous humour at the beginning and end of the diffusion interval, t, expressed in hours. K_V is similarly computed for the vitreous body. Limits of error are standard errors. The time intervals are indicated as the times, after the initial injection, between which K_A is computed; e.g. 10–20 means that K_A was computed from the concentrations in the aqueous humour 10 and 20 min. after the injection.

infinite time, the true concentrations in plasma and aqueous humour will be equal. With rapidly penetrating substances like the sulphonamides this is probably true, whilst with substances penetrating at about the rate characteristic of urea ($K_A = 14$) the existence of a flow of aqueous humour through the eye may cause a steady-state with a lower concentration in the aqueous humour than in the plasma (Kinsey & Grant, 1942). The appropriate mathematical treatment in this case has been discussed recently (Davson, Duke-Elder, Maurice, Ross & Woodin, 1949), but for the purpose of this paper the simpler treatment of Davson & Quilliam is probably adequate. The values of K_A have been computed over three time intervals, namely the first and second 10 min.,

and the whole 20 min. of penetration into the aqueous humour; only the last-mentioned period was used for penetration into the vitreous body. We may note that K_A decreases with time, an effect due to some or all of the following factors:

(*a*) *Inadequate mixing.* The equation from which K_A is computed rests on the assumption that the rate of mixing in the eye fluids is rapid compared with the rate of penetration across the barrier. With such rapidly penetrating substances as the sulphonamides this assumption is probably untrue.

(*b*) *Adsorption of the sulphonamide on to the plasma proteins.* If this takes an appreciable time, the concentration of free sulphonamide in the blood will be initially greater than its value at adsorption equilibrium.

(*c*) *Initial injection conditions.* In the first ½ min. the eye is subjected to a very high concentration of sulphonamide, since the injected material requires an appreciable time to mix with the total blood in the vascular system.

(*d*) *Adsorption in the eye.* The strong tendency of the sulphonamides to be adsorbed will be reflected in a decrease in the effective concentration in the aqueous humour at any moment.

The effects of (*b*) and (*c*) can be roughly determined by computing the values of K during the first 2 min., the succeeding 10 min., and over the whole 12 min. Mean values for a group of six rabbits with sulphadiazine were as follows:

K_{0-2}	K_{2-12}	K_{0-12}
94	40	49

The first K is too high and shows the influence of factors *b* and *c* almost exclusively. The second K is probably a little low, in that factor *a* may be beginning to make itself felt, whilst the third K is too high, since it incorporates the first 2 min. If the second K is taken as an approximately true measure of the rate of penetration, the value of K computed for the first 12 min. is then about 20% too high. The value of K computed over 20 min. (Table 2) is 42, so that it would seem that, for sulphadiazine at least, the fairest index to rate of penetration is the value of K computed over the 20 min. interval. It is impossible to assess completely the disturbing effect of adsorption in the interior of the eye so that the values of K are not strictly comparable with those obtained for substances in which this substance is not important; however, in the short intervals used here, the effect of adsorption should not introduce very serious errors.

The comparison of Tables 1 and 2 shows remarkably small differences in the values of K_A for the cat and rabbit, in spite of the large difference in volume of the anterior chambers of the two species; the parameter K_A contains an undetermined ratio of area to volume, so that either the effective area for diffusion in the cat is greater than that in the rabbit, or the true permeability constant is greater in the cat.

The order of penetration is

Sulphapyridine, Sulphanilamide > Sulphadiazine > Sulphathiazole.

Since the sulphonamides behave as weak acids at physiological pH, we may expect the rates of penetration to depend on the percentage of undissociated substance present in the blood. Figures given by Bell & Roblin (1942) are as follows: 96·6, 99·97, 20 and 38·4% respectively. The relatively high rates of penetration of sulphapyridine and sulphanilamide could be accounted for on this basis, but the fact that sulphadiazine penetrates nearly twice as rapidly as sulphathiazole suggests that more specific chemical affinities are involved. A study of ether-water partition coefficients throw little further light on the problem; the results obtained were variable, due perhaps to the chemical instability of the substances. A typical set of values, expressed as the ratio of the concentrations in ether and water, was

Sulphapyridine	Sulphanilamide	Sulphadiazine	Sulphathiazole
0·4	0·19	0·035	0·066

when the isotonic aqueous solution of sulphonamide, buffered with phosphate to pH 7·4, was shaken with ether; on grounds of partition coefficient alone, sulphathiazole should penetrate more rapidly than sulphadiazine.

If the fall in value of K_A taking place between the first and second 10 min. intervals is due in large part to failure of the substance to mix completely in the aqueous humour, we should expect the value of K_A, computed over successive intervals up to an hour, say, to continue to fall since, if mixing within the eye is only by diffusion, the effect must be progressive. In Table 3, values of K_A computed for successive intervals are recorded; it will be seen that K_A does decrease up to the 10–20 min. interval but, after this, remains fairly constant.

TABLE 3. Change in the value of K_A for sulphadiazine with penetration interval (groups of six rabbits)

Interval (min.) ...	0–2	2–12	10–20	20–40	40–60
K_A	94±6·5	40±5	27·5±4·5	29±2	30±5

DISCUSSION

The results described here show that lipoid solubility, together with specific chemical affinities, plays an important part in determining the ease with which a substance may permeate the intra-ocular fluids, since the rate of penetration of sulphapyridine, for example, is nearly twenty times that of the lipoid-insoluble sucrose which has a comparable molecular size. For such selectivity to be shown it seems necessary that a penetrating molecule should meet in its path a membrane with properties similar to the plasma membrane of the individual cell; since the vascular structures of the eye are invested with cellular membranes, we must conclude that penetration is predominantly achieved by migration through these cells.

This conclusion raises interesting problems; it is generally agreed that the endothelial lining of the anterior surface of the iris is incomplete, so that in effect the sole barrier between the capillaries, in this region, and the aqueous humour is the capillary endothelium. Thus the high degree of selectivity displayed by the barrier can only mean that the endothelium of the iris capillaries is different from that of the capillaries in the muscles, kidney glomerulus and liver. It seems safe to conclude from studies on the barrier between blood and aqueous humour, therefore, that the migration of substances from the one fluid to the other takes place by a trans-cellular route; it then becomes very difficult to separate the concepts of secretion and simple diffusion in so far as the penetration of any given substance into the intra-ocular fluids is concerned. Presumably a fluid is continuously eliminated from the cells of the ciliary epithelium; the concentration of any substance in this fluid at the moment of elimination is a matter of conjecture; it may be equal to, greater than, or less than, that in the blood, depending on the ease with which it can pass into and out of the cell, and on its involvement in metabolic processes. On its way out of the eye (through the pupil and past the surface of the iris), further exchanges with the blood are possible by way of the capillary endothelium of the vessels of the iris; and the measured rate of penetration will depend on the rate of flow, the concentration of the substance in the 'nascent aqueous humour', and the rate of diffusion from the iris.

The finding that the value of K_A settles down to a fairly constant value after 20 min. suggests that there is a mechanism for mixing the contents of the aqueous humour independently of simple diffusion. Thus the equation containing the parameter K_A is derived on the assumption that the concentration gradient between the plasma and aqueous humour is confined to the thickness of the membrane, i.e. that mixing within the aqueous humour is rapid in comparison with the penetration of the membrane. The decrease in K_A with time up to 20 min. suggests that this assumption is incorrect, so that the concentration gradient is spread over a greater depth than the thickness of the membrane, a depth which should increase with time and so cause a progressive decrease in K_A. The fact that K_A later becomes tolerably constant could most readily be explained on the assumption of a through-and-through circulation of fluid in the eye (e.g. from the ciliary body to be drained in a more remote region in the angle of the anterior chamber). This flow could bring fluid with a high concentration of sulphonamide into the eye and remove a more dilute fluid, and thus tend to compensate for the fall in rate of penetration that would occur if only diffusion processes were operative.

SUMMARY

The penetration of sulphapyridine, sulphanilamide, and sulphathiazole into the intra-ocular fluids of the cat has been measured quantitatively. The penetration of these substances, and also of sulphadiazine, has been studied in the rabbit. The generally high rates of penetration found with these sulphonamides suggest that lipoid solubility is an important factor, although the relative rates of penetration of the individual substances are not completely predictable from their ether-water partition coefficients or from the percentage of the sulphonamide that is undissociated at physiological pH.

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FURTHER OBSERVATIONS ON THE PROPERTIES OF THE CENTRAL FOVEA IN COLOUR-BLIND AND NORMAL SUBJECTS

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It has been previously reported (Willmer, 1949*a*, *b*) that, whereas the normal central fovea behaves as a colour-perceiving mechanism possessing only two, instead of the normal three, separate pathways, the foveal centre of red-green-blind subjects, whether they be protanopes or deuteranopes, behaves as though it possessed but a single pathway. This may be stated in another way. The normal person when limited to the central fovea only requires two primary colours, e.g. red and violet, with which to match all the spectral colours, and he can also match a wave-length in the region of 570 mμ. with white. These matches are only possible provided that the viewing fields fall on, and are maintained within, the central fovea. The size of the viewing fields should not subtend an angle at the eye of much more than 30′ or there is danger of information being obtained from regions outside the central fovea. Under the same conditions both protanopes and deuteranopes can be found who are able to match any colour with any other colour, including white. In other words, they are totally colour-blind when their vision is confined to this area.

Now, as already indicated, the red-green-blind individuals fall clearly into at least two classes. On the one hand, there are the protanopes, who can match red and green on a 2° field and whose spectrum is shortened at the red end. They make certain characteristic colour confusions (Pitt, 1944) and are generally considered to lack the red mechanism. Their photopic luminosity curve has a maximum in the region of 540 mμ. On the other hand, there are the deuteranopes; and they form a less homogeneous group. They again can match red and green on a 2° field and make other colour confusions which differ from those made by the protanopes, but their luminosity curves have been found to differ from subject to subject. In some, the curve is almost or quite identical with that of the normal observer; in others, its maximum is shifted towards the red end of the spectrum and falls outside the range for normal observers. In many, the curve is intermediate. Both protanopes and deuteranopes have a point in

the spectrum, which they can match with white. In protanopes this 'neutral' point generally lies within the restricted range between 485 and 500 mμ., but in deuteranopes the range may be extended to 520 mμ. or even further.

On theoretical grounds the position with regard to the central fovea may be stated as follows. If the normal observer possesses the pathways *A* and *B*, then the red-green blind might most simply be considered to possess either *A* or *B*, or he might possess both pathways for a limited distance along the neural path, but they combine together before reaching the centre at which colour perception occurs. In the first case, the receptors or their particular pathways may be literally absent, either congenitally or by atrophy; or the contribution which they make might be completely annulled by some inhibitory effect. Three types of red-green-blind person should on these lines be theoretically possible, represented by *A*, *B*, or *AB*. Moreover, in the *AB* type all gradations would be possible between *AB* and *A*, or between *AB* and *B* provided that fusion occurs before the colour centre is reached.

These ideas can be put to the test in two ways. First, the luminosity curve, for the foveal centre, of type *A* must differ from that of type *B*, and both would be expected to differ from the normal, but individuals should also be found, the *AB* type, whose luminosity curve might be identical with the normal. Moreover, if gradations occur between the *AB* type and either *A* or *B*, then intermediate curves should also be obtained. Secondly, if an individual possesses only one type of receptor and only one pathway in his central fovea, and if he adapts his eye to equal brightnesses (in terms of his own thresholds) of any spectral colours, then his threshold should always be raised to the same extent. In other words, the effects of adaptation on his foveal thresholds for different wave-lengths should be dependent on the brightness of the adapting light, but they should be independent of its wave-length at constant brightness. If, however, he possesses two types of receptor, but only one pathway, the effects of adaptation to different wave-lengths at equal brightness may be expected to lie between something approaching independence of wave-length to something approaching the pattern of adaptation presented by the normal subject, according to the relative numbers of the receptors of each type present.

The central fovea has therefore been critically studied from these two aspects in seven normal individuals and eleven red-green-blind subjects, all of whom except one could match red with green on a 2° field, and all of whom except two could match red with any other colour, or white, when the tests were made with central fixation on two 10′ fields, separated by a space subtending 10′ at the eye. In the exceptions noted above, the first could match red with all colours on the small field, and was 'green-blind' by every test on the Ishihara plates, but he was not satisfied that Ilford red (608) could match Ilford green (604) at any brightness on a 2° divided field. The other two could match all spectral colours between red and blue with red, but they affirmed that violet appeared

as a separate colour. In this connexion it is important to remember that the subjects were dark-adapted to a considerable, but reasonably constant, extent and, under these conditions, unless very reliable fixation is maintained, other elements than those in the central fovea may contribute to the sensation, and these exceptions may have arisen through inability to maintain complete fixation or through the encroachment of extra-foveal elements upon the fovea. All the colour-blind subjects tested therefore were considered to be very nearly, if not absolutely, colour-blind (monodic or monochromatic) in the central fovea. Many others have also been examined but found to be only partially colour-blind in the central fovea, though invariably they were far worse at colour discrimination in the central fovea than when tested on a 2° field. Moreover, several other subjects were tested in a preliminary series of experiments and their luminosity curves determined by a less exact method than has been used in the cases reported in this paper, but these earlier tests, including some on subjects who have also been tested by the more exact methods, gave substantially the same results. In the tests on adaptation described here, the subjects have been adapted separately to red and to violet and the effects on the red and violet thresholds obtained. These wave-lengths were chosen on the grounds that if there were only two types of receptor in the central fovea these were most likely to be stimulated separately by wave-lengths near the ends of the spectrum.

METHODS

Adaptation

A mirror was placed in front of the viewing fields used for matching the test colours in the apparatus already described (Willmer, 1949*b*). Normally this mirror reflected the field by which the fovea and immediately surrounding areas were adapted to lights of known intensity, but the mirror could be swung out of the way and the matching fields immediately brought into view. The adapting field consisted of the surface of a frosted electric bulb viewed through a lens by Maxwellian view. The brightness and colour were adjusted by insertion of appropriate Ilford Neutral or colour filters. For adaptation to red light an ordinary 100 W. tungsten gas-filled bulb was used, but in order to obtain the desired brightness of violet a mercury vapour bulb was used and the ultra-violet light cut out by means of a CS_2 filter and an Ilford 805 filter in addition to the Ilford violet filter (601). The red adapting light was mostly that obtained by means of Ilford Deep Red (609), but some of the earlier experiments were carried out with Spectrum Red (608). The adaptation field subtended about 4° at the eye and was centrally fixated. An artificial pupil of 2 mm. diameter has been used in all the adaptation experiments.

When a subject had been found who passed the suitable tests to show that he was either normal or completely red-green blind, and after he had received some previous training in foveal fixation and in matching colours and determining thresholds, he was subjected to the adapting lights. The procedure was as follows. He was first given 10 min. in the dark to bring the fovea to approximately its most sensitive state. He was next asked to view the adapting field which had first been reduced in area by means of a stop until it only subtended 20 min. at the eye, and then neutral filters were inserted until the field was reduced in brightness to threshold intensity, care being taken that the field was being viewed by central fixation only. When, after two or three trials, the threshold for this 20 min. field had been determined, it then became possible to provide adapting lights of different intensities by removing the filters in stages (density 1 each time) so that intensities varying from 1 to 10,000 times the foveal threshold could be obtained. Before adaptation proper started the

adapting field was restored to its full size (4°) by removal of the stop inserted for the determination of the threshold brightness.

For determining the 'absolute' threshold of the test light the adapting field consisted of four points of dim violet light arranged in a square of such a size that when the eye was directed to the middle of the square the points all just fell within the fovea and became invisible owing to the low sensitivity to violet in that area. Any slight movement brought them into view again and so the movement could be immediately checked and central fixation maintained ready for viewing the test fields. In some experiments a very dim red point (20′) was used for fixation. Of the test fields, the right-hand field (10′ diameter) was always illuminated by dim red light (Ilford 608) and served as a pilot for central fixation. As far as possible its intensity was maintained constant, but an increase was generally necessary to ensure steady fixation after the higher levels of brightness were used in the adapting field. The left-hand field (also 10′) was illuminated by the test light whose intensity and wave-length were controlled by filters and by movement of the light source along a calibrated scale. The subject was asked to fixate on the left-hand edge of the right-hand field (the pilot field). He first of all viewed the adapting field for 1½ min., fixating its centre, and thereafter the test fields were exposed for a period of 3 sec. every 15 sec., the adapting fields being again fixated in the intervals. During the 3 sec. he was asked to decide whether or not the test light was visible with central fixation. In the case of the violet test light the criterion mostly used was whether or not the test light could be made to disappear by direct fixation, since apart from its accidental disappearance due to head movement or the like it was not likely to disappear on account of inadequate fixation. Head movements were, of course, minimized throughout by the subject biting on a dental impression which was clamped to the bench in a suitable position determined with great precision. The setting of the lamp illuminating the test field was adjusted by the operator in such a way that the subject could have no idea of the changes in intensity, if any, which were made between each 3 sec. viewing period. By a process of irregular bracketing the threshold could be found with more accuracy than might be supposed (approximately ± 20 %).

In most cases, increasing stages of adapting brightness were used, but occasionally the experiment was performed in the reverse order, and a few subjects were tested only at zero brightness of the adapting field and at 1000 times threshold brightness. Minor modifications of this technique have been necessary or desirable from time to time, but the method described has been the usual one followed. Frequent rest periods were given and very few sittings lasted for more than 1 hr. at a time, since the requisite steadiness of fixation becomes too difficult to maintain after that time. Many unknown factors must naturally enter into work of this type, such as the degree of previous light adaptation, the degree of freshness or tiredness of the subject, and, above all, the attention he is prepared to give to the work. In spite of these difficulties, however, the results to be described will be seen to be fairly precise.

Luminosity curves

It was necessary to determine the luminosity curves for the central fovea in all the colour-blind subjects, and the threshold method was considered to be the most suitable, but, of course, this necessitated that great care be taken that strict central foveal fixation was maintained. For this purpose the subject's head position was again controlled as closely as possible by his biting on a dental impression clamped in a suitably adjustable holder. When his head was in position he could see through a 1 mm. artificial pupil a small red fixation point subtending about 2′. This point was near the centre of a small square of blue points of such intensity that they were invisible on the foveal area but they became visible as soon as central fixation was lost. The test light was then made to appear (or not) in $\frac{1}{50}$ sec. flashes at a point about 5′ away from the red fixation point and to its left. Owing to the chromatic aberration of the eye it was necessary in fixing the position of the subject's head to fix it first with a violet test light (continuously visible) and then with a red test light. If his head position was correct the field did not change its position with respect to the fixation light, but if he was not adequately centred then the two fields were found to change their positions; by changing back and forth between red and violet test lights the head position could be fixed in a reliable manner.

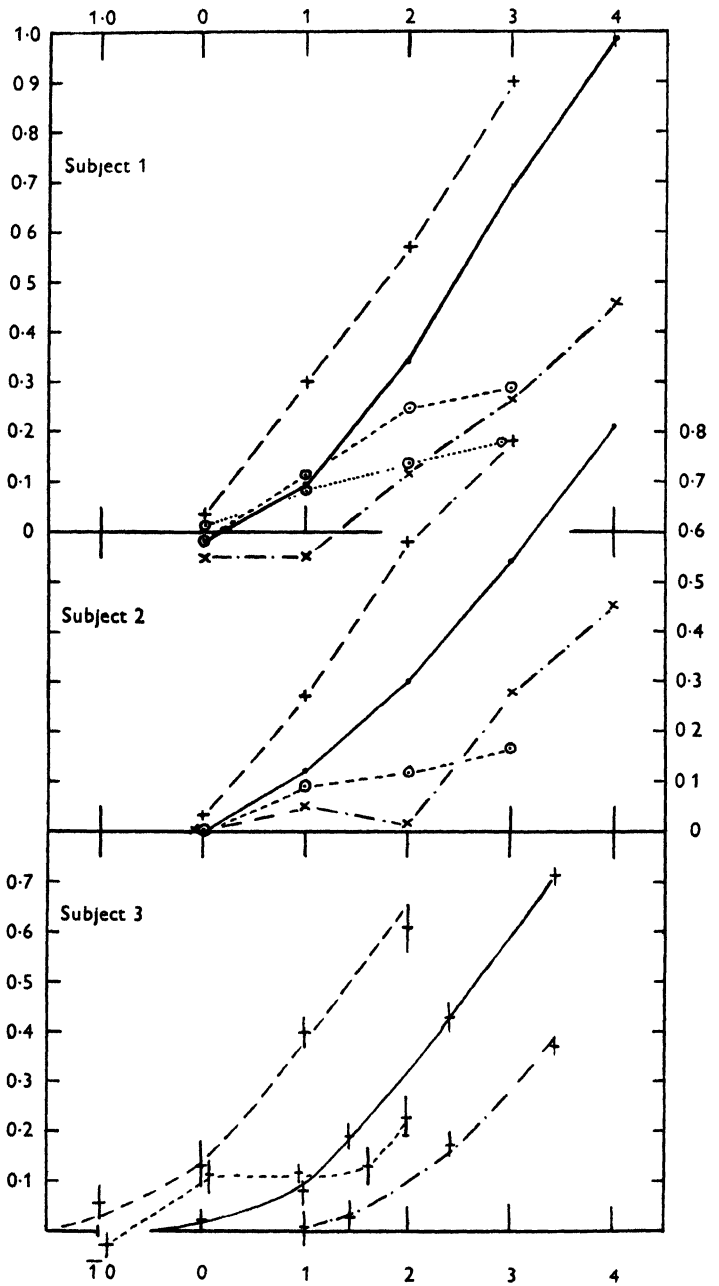

Fig. 1. For explanation of figure see p. 427.

The test lights were seen by Maxwellian view, and the field was delimited by a stop whose aperture subtended an angle of about 8′ at the eye. The field was illuminated by means of a Hilger Monochromator, and the light source was provided by a ribbon filament lamp (6 V. 18 A.) run at 4·4 V. from batteries which were being simultaneously charged. Intensity control was carried out by calibrated neutral filters, and a pair of polaroids placed in the exit beam. The calibration of the energy of the spectrum and the transmission of the polaroids was kindly carried out by Dr L. C. Thomson by means of a calibrated electron multiplier photocell. The duration of the flash, which was 12 msec., was determined by an adjustable rotary pendulum shutter, and has remained constant in these experiments. This short flash was used because it was hoped that by this means there would be no possibility of any eye movements as the result of the flash, so that if the subject's eye was well fixated there was no possibility of the test light falling outside the so-called rod-free area.

The subject was in complete darkness during the experiment and no readings were taken until he had been in the dark for 15 min. Each session lasted about an hour and for each curve the points were repeated four or five times, and the scatter of the points indicated a variation in the determined value of the threshold equivalent to a factor of about 2. The readings taken during the first session nearly always indicated a lower sensitivity than those found subsequently; some sharpening of the criterion for visibility always occurred. It also seemed to be evident that some of the later variation was due to an actual change in sensitivity of the eye from day to day. It must, however, be remembered that errors in centration and the Stiles-Crawford effect (Stiles & Crawford, 1933) may also contribute to the variability.

The curves were plotted between 480 and 700 mμ. Only a few readings were taken at wavelength shorter than 480 mμ. because the variable quantities of macular pigmentation in different subjects make estimation of the sensitivity in that region of doubtful value as an index for the sensitivity of the receptors.

RESULTS

Adaptation

All the experiments described here concern the manner in which the thresholds for lights from the spectral extremes are changed by adaptation to the same light as the test light or to the opposite light. The effects of red adaptation on red violet thresholds, and of violet adaptation on violet and red thresholds, have been determined. If, as seems fairly clear now, the foveal centre of a normal subject only possesses two receptors, then these lights from the spectral extremes might be expected on general principles to stimulate one receptor considerably more than the other, at least at low intensities, though it is possible that at one

Fig. 1. Diagrams showing the extent to which the thresholds for red and violet are altered by adaptations to different intensities of red and violet, in three normal subjects 1, 2 and 3. +----+, effect of violet adaptation on violet threshold; ·————·, effect of red adaptation on red threshold; ⊙.....⊙, effect of violet adaptation on red threshold; ×—·—·—×, effect of red adaptation on violet threshold. Ordinates: logarithm of the multiples of the original threshold with no adapting light. Abscissae: log intensity of the adapting light; 0 = intensity of the adapting light corresponding to the threshold for a 20′ centrally fixated field. At this intensity the field appears coloured. At intensity 1·0 the violet field (4°) became almost colourless. The red field was below threshold at this intensity. In subject 3 the vertical strokes indicate the standard error of the mean of about thirty readings for each point. The technique of finding the threshold in subject 3 was slightly different from that in the other two subjects. The subject (E.N.W.) altered the intensity himself and judged the threshold within 20 sec. of removing the adapting light, after an adaptation time of 3 min.

end of the spectrum the two receptors might have similar sensitivities and so be stimulated more or less equally.

Normal subjects. The curves expressing the pattern of adaptation in three normal subjects are shown in Fig. 1, where the extent to which the threshold is raised by adaptation to lights of increasing intensity is plotted against the logarithm of the intensity of the adapting light, in terms of the threshold intensity for the subject. The method used for one of the three subjects was slightly different, and either because of that, or for some other reason, the effects of the adapting lights seem to start at lower intensities, but essentially the same pattern is obtained.

There are several points of interest about this pattern, though it is not the purpose of this paper to discuss their possible significance. In the first place, though the red and violet adapting lights were of the same strength to the subject, as determined by foveal fixation on a 20′ field, yet the violet affected the violet threshold to a greater extent than the red affected the red threshold. In fact the violet light seemed to affect the violet threshold at intensities below the foveal threshold. It is, of course, important to remember that 'foveal threshold' is a variable quantity according to the size of the field and may be well above the actual threshold for the receptors, which, however, may not be able to evoke an adequate sensory response unless considerable summation occurs. It has been shown elsewhere (Willmer, 1950) that the relationship between area and brightness is the same for red as it is for violet within the foveal area, so that it does not appear likely that the difference between red and violet is to be sought along these lines. Another point to remember is that the subjective impression, when comparing the brightness of the 4° adapting fields, is that the violet field is very much the stronger, at least when it is first compared, but this apparent brightness is very largely due to the stimulation of the parafoveal rods and the Purkinje shift. It is also possible that the stimulation of the peripheral rods may contribute to the factors determining foveal thresholds. Nevertheless, in a few experiments where the red and violet adapting lights were equalized in intensity at a higher level by direct heterochromatic matching the violet adaptation produced the greater effects.

It is clear that the effect of the violet adapting light on the threshold for violet is, at all intensities, greater than that of violet on red, or of red on either violet or red. The three curves expressing the effects of violet on violet, red on red, and red on violet are in all probability much the same curves, but shifted along the intensity axis. At least, this seemed to be so in the case of the author's eye where the curves are based on a much greater number of observations. The curves for observer 3, in Fig. 1, are all the same curve and it is seen that they satisfy the data reasonably well. On the other hand, the curve showing the effect of violet adaptation on a red test light seems to be somewhat different. Roughly speaking it has about half the slope of the other curves and in some

observers there is even some suggestion of an early rise, a plateau and a second rise. The meaning of this difference is at present obscure and must await further research, but the important conclusion now is that in the normal subject there is a definite pattern of the effects of adaptation to red and to violet lights and that this pattern supports the view that the normal fovea possesses two receptors, one of which is more stimulated by violet light than it is by red light, and the other more by red than by violet. This may not be all that can be deduced from the shapes of these curves but it is the essential point for the present argument.

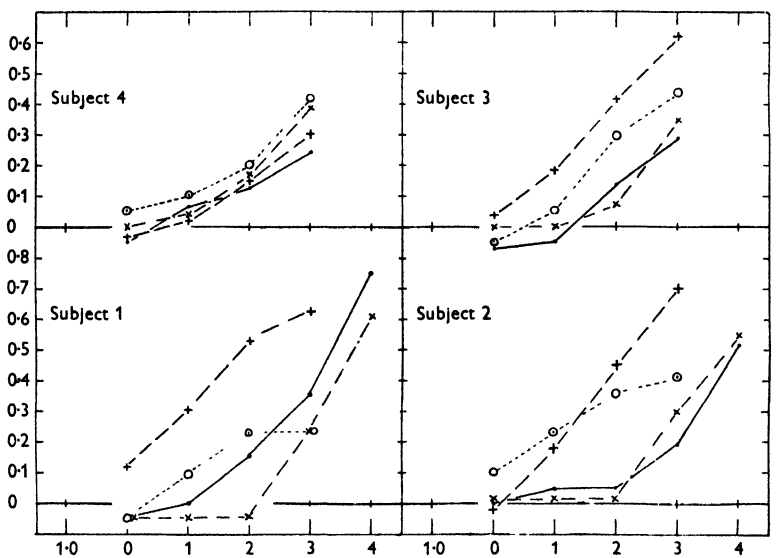

Fig. 2. Diagrams showing the extent to which the thresholds for red and violet are altered by adaptation to different intensities of red and violet, in four deuteranopes. All conventions as in Fig. 1. Note that subject 1 has a pattern indistinguishable from the pattern for the normal subject (see Fig. 1). In subject 4, however, it is doubtful whether the differences between the curves are significant.

Deuteranopes. If now the same tests are performed on the red-green-blind subjects who show no insensitivity to red, i.e. on the deuteranopes, the results are sometimes very different from, and sometimes they are closely similar to, those obtained on the normal observer. Fig. 2 shows a series of sets of curves obtained on four different observers, and, besides those shown, other intermediate types have also been obtained, so that there is clearly a graded series from observer 1 to observer 4. Observer 1 could not be distinguished from the normal observer on the basis of this test, yet he is totally colour-blind in the foveal centre. On the other hand, observer 4, also totally colour-blind in the foveal centre, shows a pattern of adaptation in which the curves cannot be distinguished from each other. For this subject and within the limits of experi-

mental error, red and violet lights at the same brightness seem to have the same capacity for raising the threshold for both red and violet.

These results clearly point to the conclusion that observer 4 is totally colour-blind in the foveal centre because he only possesses one type of receptor and one pathway. At the opposite end of the scale, observer 1 shows evidence of possessing the same receptors and perhaps even the same pathways as the normal person, but somewhere the paths must fuse before they reach the 'colour centre'. Among the deuteranopes therefore there are many different grades which extend on the one hand from observers whose pattern of adaptation is indistinguishable from the normal (deuteranopes, type I), to those, on the other hand, whose pattern of adaptation suggests the presence of but a single type of receptor (deuteranopes, type II). In other words, if the normal eye possesses receptors A and B which transmit their effects along two pathways a and b, the eyes of the deuteranopes tend towards one of two extremes in which, on the one hand, these receptors have a common path (ab) or, on the other hand, one of the receptors and its pathway is missing (say B and b). These two extremes may be conveniently labelled deuteranope type I, in which the adaptation pattern is nearly normal, and deuteranope type II in which the adaptation pattern suggests a single type of receptor.

Table 1 shows the extent to which the thresholds are raised for various observers by adapting lights of one thousand times the threshold intensities as determined for the respective subjects. The figures include some for observers in whom it has not been possible to obtain full curves at different intensities, and it will be seen that the 'pattern' for the deuteranopes conforms to the one deduced from the observers for whom full curves have been obtained.

Protanopes. Table 1 also includes figures for two protanopes, i.e. red-green-blind individuals who are relatively very insensitive to red. Now these observers again show a characteristic pattern. In them, the red threshold is raised to approximately the same extent by either red or violet, and similarly the violet threshold is raised equally by red or violet adapting lights, but the extent to which the two thresholds are raised is somewhat different, and this difference, though small, has been noted rather constantly and is probably outside experimental error. It is difficult to see how such an effect could be brought about, but it could occur if the adaptation, or some of it, is in the nerve pathway as well as in the receptor. Thus it is possible to imagine that there are two receptors present in the protanope, one mainly sensitive to the longer wave-lengths and the other sensitive to the blue end of the spectrum. If the latter showed some degree of photochemical adaptation while both receptors produce adaptation in the neural pathway, then the neural adaptation should be caused equally by the red and violet adapting lights, but photochemical adaptation in the 'violet' receptor would raise the threshold to violet more than to red, though presumably violet would be more effective in causing the photochemical adaptation than

red and this does not seem to be so. The fact, however, that red and violet adapting lights produce equal effects on the threshold for red, and equal effects on the threshold for violet suggests very strongly that there is only one neural pathway though there may be more than one receptor. This, of course, agrees with the finding that the protanopes are completely colour-blind in the foveal centre. Of the two tested here, one was completely colour-blind in the foveal centre, the other could distinguish violet from other colours, but these other

TABLE I. The effects of adapting to red or violet on the central foveal thresholds for red or violet. 'Red on violet', etc., means the effect of adaptation to *red* light *on* the *violet* threshold.

The numbers in columns 3 to 6 indicate the logarithm of the number of times the threshold is raised by adaptation to a light of intensity 1000 × threshold, i.e.

$$\left[\log \frac{\text{Threshold after adaptation to red (or violet) light at } 1000 \times \text{threshold}}{\text{Threshold after adaptation to darkness}}\right].$$

In columns 7 and 8, if the violet and red receptors were one and the same, then one would expect the differences to be zero. Note that for deuteranopes of type II this is approximately true.

(1) Type of subject	(2) Subject no.	(3) Red on red	(4) Red on violet	(5) Violet on red	(6) Violet on violet	(7) Difference between red on red and red on violet	(8) Difference between violet on violet and violet on red
Normal	1	0·62	0·30	0·35	0·90	0·32	0·55
Normal	2	0·31	0·01	0·18	0·45	0·30	0·27
Normal	3	0·47	0·35	0·04	0·64	0·12	0·60
Normal	4	0·43	1·92	0·16	0·38	0·51	0·22
Normal	5	0·44	0·15	0·08	0·59	0·29	0·51
Normal	6	0·59	0·27	0·17	0·79	0·32	0·62
Normal	7	0·63	0·26	0·30	0·71	0·37	0·41
	Mean	**0·50**	**0·18**	**0·18**	**0·64**	**0·32**	**0·46**
Deuteranopes Type I	1	0·29	0·00	0·24	0·45	0·29	0·21
	2	0·34	0·10	0·25	0·71	0·24	0·46
	Mean	**0·32**	**0·05**	**0·25**	**0·58**	**0·27**	**0·33**
Intermediate	3	0·21	0·23	0·32	0·67	−0·02	0·35
Intermediate	4	0·50	0·46	0·30	0·82	0·04	0·52
Intermediate	5	0·43	0·57	0·55	0·74	−0·14	0·19
Intermediate	6	0·30	0·36	0·43	0·63	−0·06	0·20
	Mean	**0·36**	**0·40**	**0·40**	**0·72**	**−0·04**	**0·32**
Type II	7	0·24	0·40	0·42	0·30	−0·16	−0·12
Type II	8	0·44	0·39	0·41	0·43	0·05	0·02
Type II	9	0·33	0·38	0·37	0·30	−0·05	−0·07
	Mean	**0·34**	**0·39**	**0·40**	**0·34**	**−0·05**	**−0·06**
Protanopes	1	0·29	0·52	0·43	0·59	−0·23	0·16
Protanopes	2	0·27	0·40	0·23	0·34	−0·13	0·11
	Mean	**0·28**	**0·46**	**0·33**	**0·46**	**−0·18**	**0·13**

colours could all be matched among themselves, and with white, providing that the brightnesses were made equal. Further work is, therefore, clearly needed in order to determine exactly how the protanopes behave with respect to these adaptation tests. So far as the available results show, protanopes behave

differently from the normal observer and probably differently from the deuteranopes of type II, though it is not possible to say definitely that only one type of receptor is present.

Luminosity curves

Protanopes. Further information on the properties of the foveal centre can be obtained from the luminosity curves for this area. If, for example, as the

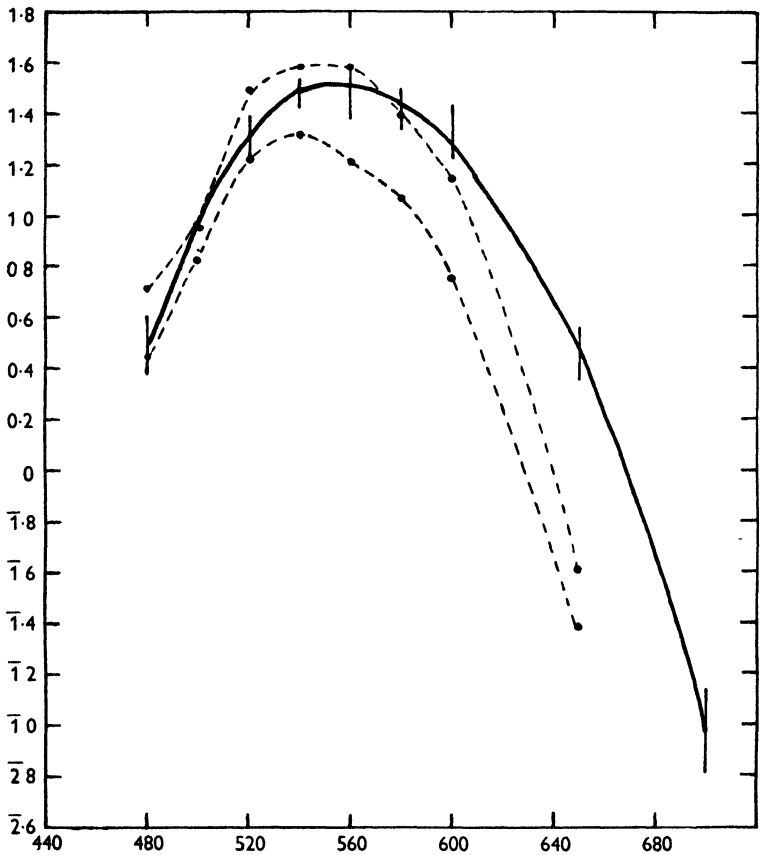

Fig. 3. Luminosity curves for the central fovea of the normal subject, and for two protanopes. Equal energy spectrum. ———, normal luminosity curve (mean for five observers). - - - - -, luminosity curves for the protanopes. The vertical lines on the normal curve indicate the range of variation among the normal subjects. The curve for each subject is the mean of five readings for each point, each of the five readings being taken on a separate day. Ordinates: logarithm of luminosity (arbitrary units). Abscissae: wave-lengths (mμ.).

experiments on adaptation indicate, some of the colour-blind subjects have only one type of receptor in the foveal centre, then the luminosity curve for this region of such subjects must measure the spectral sensitivity of the single type of receptor. When this curve is determined for the protanopes, it differs greatly from that of the normal foveal centre. It has its maximum at about

540 mμ. and indicates a conspicuous loss of sensitivity to all wave-lengths towards the red end of the spectrum as compared with the normal (Fig. 3). From a series of preliminary observations on a group of protanopes previously investigated it appears that there is very great variation in the shape of the

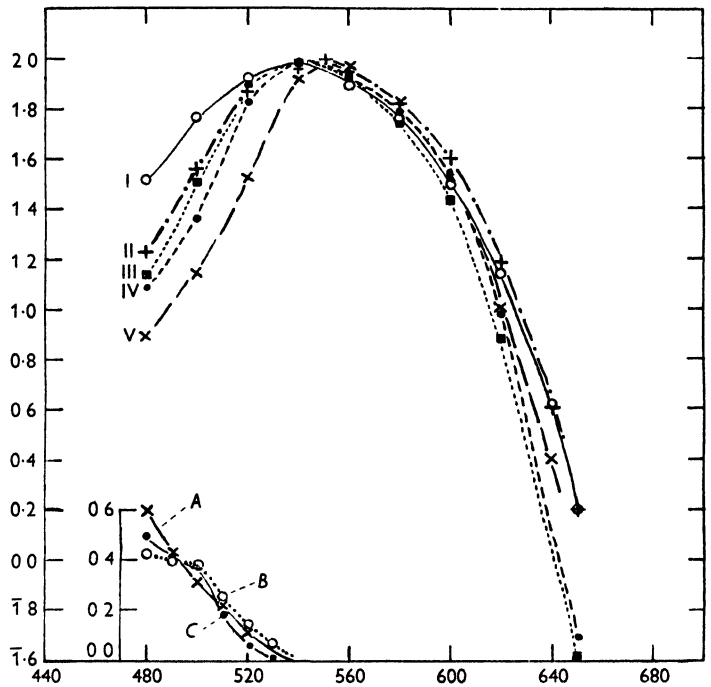

Fig. 4. Luminosity curves for five protanopes showing the comparative uniformity of the curves towards the red end of the spectrum and the diversity towards the violet end. The curves have all been shifted along the vertical axis to have the same maximum. Equal energy spectrum. Data for some of these curves supplied by Dr L. C. Thomson and obtained by a different technique. Curve I, though belonging to a subject who is not quite completely monochromatic in the central fovea, has been used as the standard curve for an estimation of the extent to which the amount of macular pigment accounts for the differences between the curves in the blue region of the spectrum. Curve *A* (× — ×) represents the density of macular pigment as determined by Wald (1945) from differences between the photopic luminosity curve for the periphery and that in the fovea. Curve *B* (○ · · · · ○) represents the density of macular pigment as deduced by subtracting the mean of the four curves (II–V) from curve I. Curve *C* (● – – ●) represents the density of macular pigment as deduced by subtracting the ordinates of the mean curve for the two protanopes whose curves are shown in Fig. 3 from the visual purple curve, as determined by Donner & Granit (1949) for the cat, but moved along the spectrum to fit the protanope's curve. (Both curves corrected for quanta.) (See Text, p. 441.)

curve in the blue part of the spectrum (Fig. 4). Sometimes the sensitivity is considerably above that of the normal observer (Dr Thomson has reported a case to me in which the sensitivity was nearly ten times greater than the normal at 450 mμ.), and in others the sensitivity is normal or even rather

below the normal. How far these observations are due to actual differences in the sensitivities of the receptors, and how far they are due to variations in the distribution of macular pigment, or to the encroachment of low threshold rods into the foveal area, or merely to inadequate fixation it is at present difficult to decide. The position of the maximum and the shape of that part of the curve which extends from the maximum to the red end is much less variable, and can be regarded as correct with some degree of certainty. If in Fig. 4, where the curves have all been brought to the same maximum, the highest curve in the blue (curve I) region may be regarded as representing the greatest sensitivity because the observer has least macular pigment (an assumption for which there is no direct evidence) and the other curves indicate lowered sensitivity because of greater amounts of macular pigment, then the loss in sensitivity can be estimated for the different subjects by taking the difference between this curve and the other curves of Fig. 4. These differences will represent the density of the supposed macular pigment. The density of the pigment, deduced by subtracting the ordinates of the mean of the four lower curves from those of curve I, is shown in Fig. 4, where the values are compared with the densities of macular pigment as deduced by Wald (1945) for the corresponding region of the spectrum. Wald's figures were obtained in a similar manner by comparison of cone sensitivity (photopic luminosity curve) in the periphery of the retina with that in the fovea. Clearly the two sets of figures based on different data are of the same order of magnitude and follow much the same trend. Wald found that the absorption by macular pigment in different subjects may vary between 0 and 90 % at 436 mμ., so the agreement is well within the bounds of probability and it may be tentatively concluded that the differences in the form of the sensitivity for the different protanopic observers in this region of the spectrum is at least partly explained by differences in pigmentation. In addition to this there are, of course, the absolute differences of sensitivity between different observers to be explained. These differences are not so apparent in Fig. 4 where all the curves have been arbitrarily given the same maximum. Fig. 3, however, indicates the order of magnitude of the variation which may occur between observers.

Deuteranopes. In general, the luminosity curves for the deuteranopes differ less from the normal than do those of the protanopes. In fact, some are indistinguishable from the normal. In Fig. 5 three curves are plotted. One is for five normal observers. The second is for three deuteranopes in whom the red adaptation light produced almost the same effects on red and violet thresholds, and in whom violet adaptation also produced equal effects on the two thresholds, i.e. for those deuteranopes (type II) in whom the adaptation pattern most clearly differed from that of the normal. This curve confirms some preliminary observations (Willmer, 1949*a*) and shows a maximum between 570–580 mμ. with a significant difference from the normal towards the blue end of the spectrum. The difference between the curves for the deuteranope type II

and the normal subject conceivably might be due to differences in pigmentation in the retina and ocular media, but such evidence as that of Wald (1945) and the earlier work of Abney (1895) and Sachs (1891) all suggest that such effects

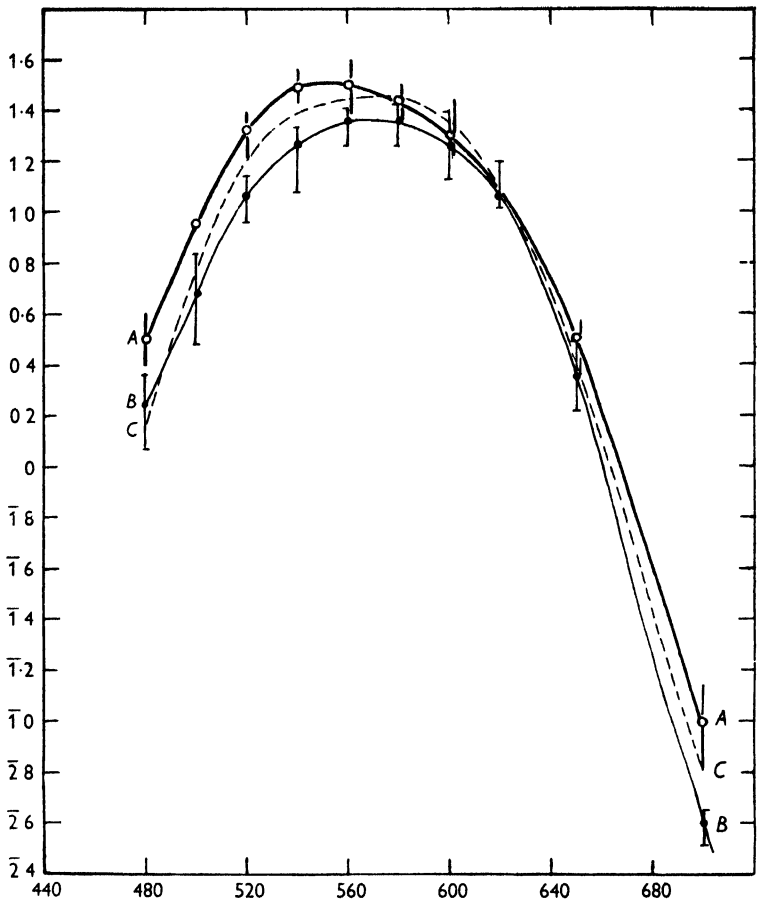

Fig. 5. Luminosity curves for normal and deuteranopic subjects. Equal energy spectrum. *A*, mean normal luminosity curve. The vertical lines show the range of the mean curves for five observers. *B*, mean luminosity curve for three deuteranopic observers for whom the adaptation pattern is like that shown in Fig. 2, no. 4, i.e. observers in whom the foveal centre apparently possesses but one type of receptor. The vertical strokes indicate the extremes. *C*, mean luminosity curve for three deuteranopic observers for whom the adaptation pattern is like that shown in Fig. 2, no. 3. Ordinates: logarithm of sensitivity—arbitrary units. Abscissae: wave-lengths (mμ.).

become very small or negligible at wave-lengths longer than 520 mμ., while the difference between these two curves does not disappear till about 580 mμ. The third (dotted) curve is for three deuteranopes in whom the adaptation pattern was similar to that in Fig. 2, no. 3. These observers can be considered as

tending towards deuteranopes of type II, but there is still an indication of another receptor. In other words, they are 'intermediate', and this adjective could also be applied to the position of their luminosity curve between that of the normal and that of the deuteranopes, type II. The luminosity curves for

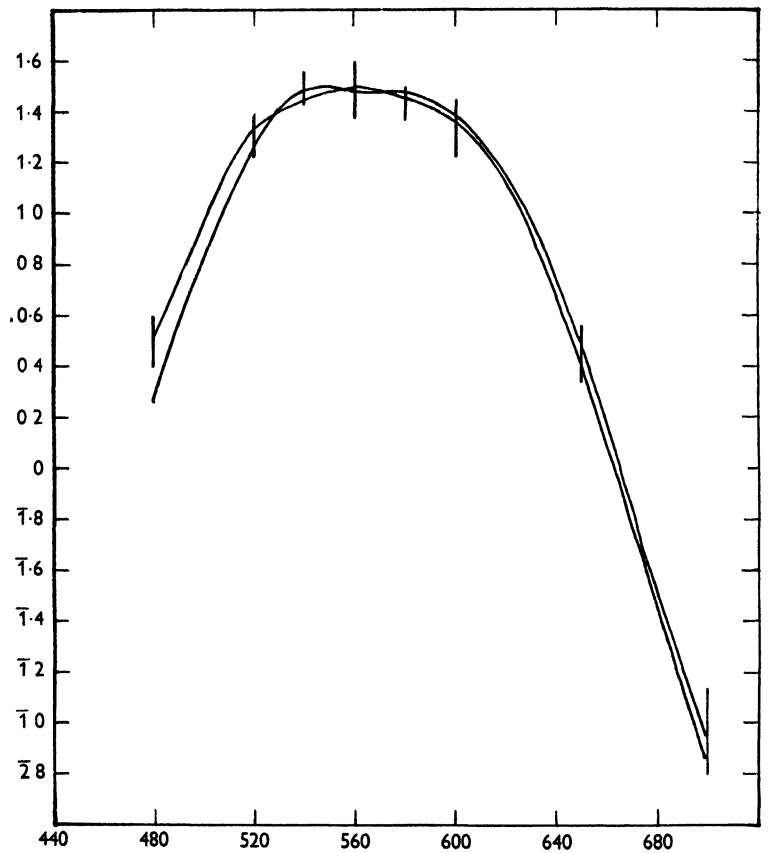

Fig. 6. Luminosity curves of two deuteranopes (type I) whose pattern of adaptation does not differ significantly from that of the normal observers (see Fig. 1, no. 1). The vertical lines indicate the range of variation among the five normal observers. Equal energy spectrum. Ordinates: logarithm of sensitivity—arbitrary units. Abscissae: wave-lengths (mμ.).

two observers in whom the adaptation 'pattern' did not differ appreciably from the normal (deuteranopes, type I, see Fig. 2, no. 1) are not significantly different from the normal luminosity curve (Fig. 6).

It appears, therefore, that just as the adaptation patterns tend towards two extremes, so the luminosity curves also divide the deuteranopes in a similar manner.

There appears to be no consistent difference in general sensitivity between deuteranopes and normal observers. While some deuteranopes, belonging to

both types, have been found with very low sensitivity as compared with the normal, in others, again belonging to both types, the sensitivity may be well within normal limits and in some cases even slightly above. Thus there is no clear support for the view that deuteranopes are necessarily less sensitive to light than normal observers, though in many cases they undoubtedly are. An important experimental error may easily creep into determinations of this type, for it has been found that when a subject first begins to determine thresholds he invariably obtains higher values (indicating lower sensitivity) than he will subsequently do. Owing to the difficulty of obtaining colour-blind observers for unlimited tests it is often necessary to take only a few series of readings, whereas the readings so obtained are often compared with those of a normal observer well trained in the technique. This comparison may introduce quite a considerable error.

Subjective colour impressions

Although very little weight can be attached to the data acquired in this way, there are some interesting observations which are worth recording with regard to the names given to the colours seen, or imagined, during the determination of the luminosity curve by the flash method. All the flashes were made very near to the threshold level and the subject was unaware of the wave-length employed. Under such conditions most of the colour-blind subjects reported all the flashes as colourless or very pale blue, or pale blue-green. A few when tested on violet reported it as violet, though this generally occurred in those subjects who found it possible to distinguish violet from other colours on the small field. One deuteranope (in the terminology of earlier days, 'green-blind') reported all the flashes as green! Since, however, he could match green and white this was obviously not very significant. It became quite clear that the colour-blind subjects were unable to distinguish the colours in these short flashes and they behaved in a manner consistent with vision by one pathway only. They behaved as the normal person does when his vision is restricted to the rods in twilight vision.

An interesting point thus emerges. When the observer has only one receptor and one pathway, as the deuteranope of the second type appears to have, that receptor, which in this case might be described as the red receptor, does not give him the sensation of red, but a sensation of 'no-colour'. Similarly, when the other type of pathway found in the deuteranope of type I is responding alone, then, again, only a 'colourless' sensation is perceived. Presumably, colour sensations must derive from differences in response between two or more pathways, and not as the result of the response along a single pathway when this cannot be compared with the response along another or other pathways.

In this connexion the colour impressions obtained by the normal observer are also worthy of note. Colours at the red end of the spectrum were mostly reported as red, but as the wave-length became shorter, a number of flashes

were reported as colourless. In the orange region a few flashes were reported as green. In the region from 570 to 590 mμ. flashes were reported as orange, colourless, and green, in proportions varying with the wave-length. From 570 to 480 mμ. green or blue-green was the answer which preponderated more and more first over orange and then over colourless. Now the normal person matches a wave-length in the region of 570 mμ. with white under conditions of foveal fixation. Thus the colourless sensation here again probably depends on there being no difference of response in the two pathways. Under the 'flash' conditions, perhaps in accordance with Hartridge's cluster theory (Hartridge, 1947), the stimulus may sometimes be more effective in initiating impulses in one receptor type rather than in the other, and these unbalanced responses give rise to the two 'coloured' sensations, green or orange, which the normal observer may receive from a flash of light which should be colourless. The degree of unbalance will presumably be reflected in the saturation of the flash.

DISCUSSION

The experimental observations recorded in this paper are consistent with the view expressed in the introduction and which may be summarized as follows. The central fovea of the normal subject possesses two types of receptor and two independent pathways. It should be noted, however, that the two pathways need not necessarily each correspond to the sensitivity of one of the receptors, but either or both of them might pass on effects set up by both receptors in different proportions. Indeed, some such idea may have to be invoked in order to account for the characteristic adaptation 'pattern' to red and violet displayed by the normal observer (see Fig. 1). For example, if the receptors themselves are labelled A and B, then the pathways may relay from A and B separately, but more probably they relay from some combination like AB and B. Exactly how the effects combine in AB can only be determined by further research; but it is possible that AB may represent pathways in which A adds its effects to B, in which A detracts from the effects of B, or vice versa. All such arrangements would be consistent with a dichromatic fovea, for these different pathways would not be independent pathways—they would all essentially depend on the sensitivities of the two primary receptors A and B.

If now the protanope is truly monochromatic or monodic in his central fovea, and if B be considered as the receptor covering the red end of the spectrum, he might very well not have this separate pathway, but rely on the A, or more probably the AB receptor pathway only. This would be consistent with his lower sensitivity in the red; moreover, the complexity of his adaptation pattern suggests that his single pathway is not a simple one, so that it is more likely to be AB.

The deuteranope whose adaptation pattern resembles the normal pattern and whose luminosity curve is indistinguishable from the normal must clearly

have the same receptors as the normal and it must be supposed that their effects are combined together before reaching the 'colour' centre. In other words, if the normal observer possesses *AB* and *B*, then this type of deuteranope possesses these receptors also, but the pathway gives 'pooled' information (*AB* and *B*) only. This is consistent with Pitt's (1944) observations and deductions from colour mixing data from which he suggests that deuteranopes have their red and their green pathways fused.

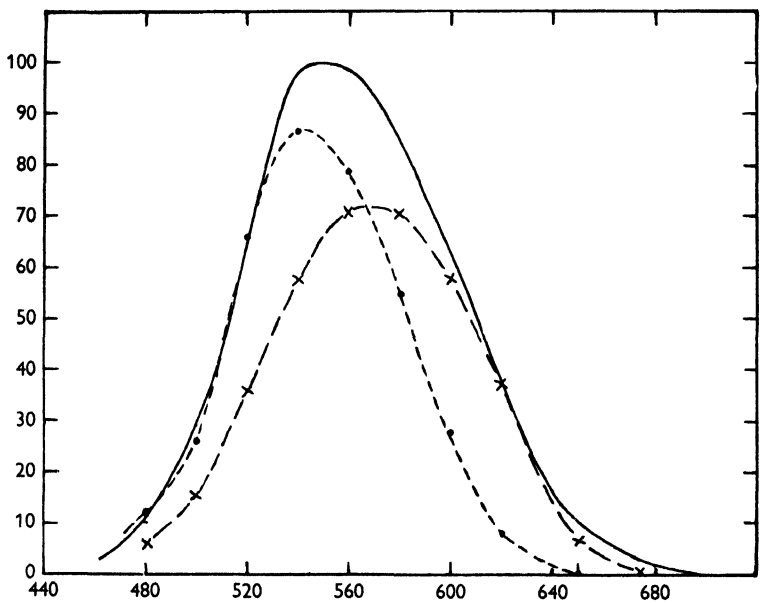

Fig. 7. Luminosity curves for the central fovea of normal subjects. (————), deuteranopes, type II (— — —), and protanopes (- - - - -). Equal energy spectrum. Ordinates: sensitivity, as percentage of the maximum. Abscissae: wave-length (mμ.).

On the other hand, the deuteranope of the other type has his maximum sensitivity moved towards the red end of the spectrum (see Fig. 7), and his adaptation pattern is simple, all lights of equal brightness to him exerting equal effects, so that it is tempting to believe that the limit in this direction is reached when receptor *B* alone is present. If this is so, then it means that the luminosity curve for this type of deuteranope represents the spectral sensitivity of receptor *B*. In this connexion it is interesting to observe its resemblance to the curve expressing the sensitivity of the red mechanism according to Stiles (1939) and that the curve has a very similar form to that of visual purple, but is displaced along the spectrum towards the red (Fig. 8). Krause (1942) has called attention to the fact that when the length of the conjugated chain is increased in certain dyes, similar in structure to the carotenoid of visual purple, then the absorption spectra of the resulting dyes may remain unchanged in form but

shifted towards the red end of the spectrum. The sensitive substance of receptor B may therefore be a similar modification of visual purple. Moreover, the spectral sensitivity of receptor B is not very different from the curve recorded by Granit (1942) as the sensitivity of the photopic receptor (cone) in the pigeon, and its maximum lies in the same region as the probable maximum for the one photo-sensitive pigment which has been extracted from cones, namely

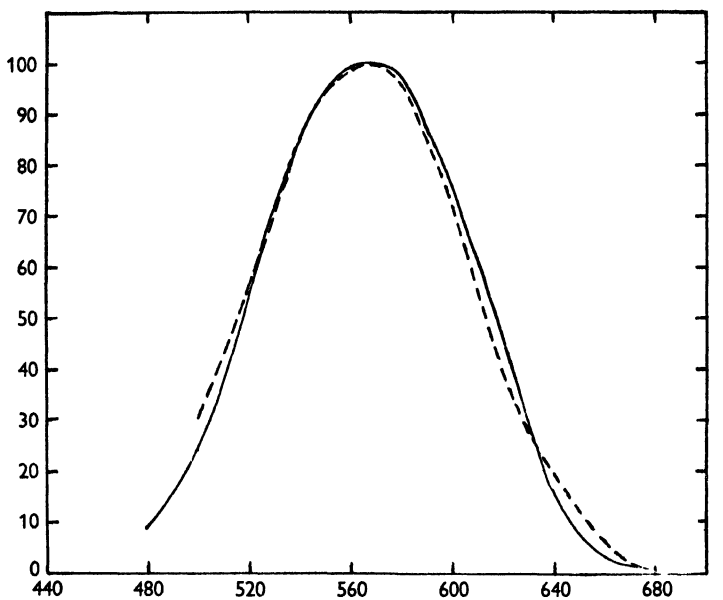

Fig. 8. Luminosity curve for the deuteranope, type II (————), compared with the luminosity curve for the visual purple receptors in the cat's retina as recently determined by Donner & Granit (1949) (- - - - - - - -). Both curves corrected for quanta, and the visual purple curve moved along the spectrum to have the maximum at 570 mμ. Ordinates: sensitivity as percentage of the maximum. Abscissae: wave-length (mμ.).

iodopsin, the pigment extracted from the retina of the chicken by Wald (1937) and also by Bliss (1946). All these observations may perhaps point to the idea that receptor B is none other than the human cone.

If this is so it remains to be seen whether receptor A is another variant of visual purple, or is visual purple itself. All that can be said at the moment is that if the protanope depends on receptor A only, then receptor A is not solely dependent on visual purple because the protanope has his maximum sensitivity at 540 mμ. and not near 507 mμ. But if, as seems probable, the second receptor of the normal observer, and the remaining receptor of the protanope, is a composite receptor then A may well contain visual purple. Although insufficient data are at present available, it is interesting that the form of the protanope's sensitivity curve again resembles that of the visual purple absorption curve, though differing in its position in the spectrum, and being somewhat too low in

the short-wave end of the spectrum. This resemblance to the visual purple curve could arise either if the receptor again depended on some simple chemical modification of visual purple which caused its absorption curve to be shifted along the spectrum towards the red end, or if the sensitivity curve resulted from the combined action of two receptors each of which depended on a substance whose sensitivity curve had the same shape as that of visual purple, but each of which had a different point of maximum sensitivity. Porphyropsin, the receptive substance found in certain fishes, is almost certainly a modification of rhodopsin, and it has its maximum sensitivity at about 522 mμ. It is, indeed, conceivable that the receptor of the protanope might be sensitized by this substance, but its maximum is not at the right point nor is there as yet any evidence either for its occurrence in the mammalia, or for the presence of the related substance vitamin A_2 in man or mammals.

It has been pointed out that the protanope's sensitivity curve differs from the visual purple curve in a systematic way in the blue part of the spectrum. This difference begins at about 530 mμ. and steadily increases towards the blue, and this is exactly the sort of change in sensitivity which would occur in a receptor dependent on a visual purple variant (max. 540 mμ.) if it were screened by macular pigment. Now it has already been shown that much of the variability of protanopic sensitivity in the blue could be explained in terms of differing quantities of macular pigment. So it is relevant to see whether the differences between the protanope's curves and the visual purple curve indicate the same spectral distribution of absorption by macular pigment as is indicated (1) by the variability among the protanopes themselves and (2) by the difference between peripheral and central cone sensitivity which was used by Wald to estimate the absorption by the pigment. Now when the protanope's luminosity curve is plotted on a logarithmic scale, and the visual purple curve is superimposed and given the same maximum, then the difference between these curves should give, if the foregoing reasoning is sound, the 'density' of the macular pigment. The result is plotted in Fig. 4, curve *C*, and is clearly of the same kind as curves *A* and *B*, which are the previous estimates of the absorption by macular pigment. Thus, allowing for macular pigment, there are strong indications that the spectral sensitivity curve of the protanope's receptor is fundamentally similar to that of a visual purple receptor, but shifted along the spectrum towards the red, and in this way it resembles the spectral sensitivity curve for the deuteranope, type II, but it is not displaced so far. For this there seem to be two possible explanations. The curve for the protanope would result either if the receptor depended on a chemical variant of visual purple, or if the curve were itself the result of combined activity of two receptors such as a visual purple receptor and the receptor *B*. For example, it is possible that the response from a ganglion cell might be an average response of the two or more receptors which play upon it. Now the response from a single receptor is

roughly proportional to the logarithm of the intensity of the light acting upon it. Therefore, the response of the receptor to different wave-lengths is roughly proportional to the ordinates of its spectral sensitivity curve when this is plotted on a logarithmic scale. The ordinates may thus be looked upon as a measure of the nerve impulses set up at the different wave-lengths by

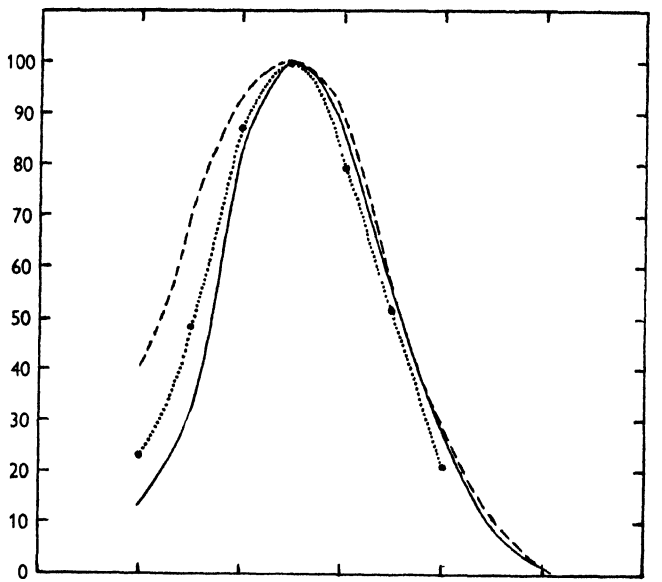

Fig. 9. Comparison between the sensitivity curve of the protanope (————), the sensitivity curve of a visual purple receptor shifted along the spectrum to have its maximum at the same point (– – – –), and the curve expressing the spectral sensitivity of a ganglion cell whose response is a mean response between that of an ordinary visual purple receptor and that of a receptor having the sensitivity of those present in the fovea of the deuteranope type II. (.....). Spectrum of equal quantum intensity. Ordinates: sensitivity as percentage of the maximum. Abscissae: wave-length (mμ.).

a spectrum of equal quantum intensity. When two receptors are acting, each will therefore respond throughout the spectrum in proportion to the ordinates of its logarithmically plotted sensitivity curve, and the response from the ganglion cell at any particular wave-length would thus, on the above assumption, be the mean of these ordinates. Naturally, it might equally well be the sum, or the difference, or almost any other combination for all the direct evidence that exists, but it is perhaps significant that the curve expressing the mean between the ordinates of the visual purple curve and the sensitivity curve of the deuteranope, type II, when corrected for the absorption by macular pigmentation, is almost identical with the spectral sensitivity curve of the protanope (Fig. 9).

However this may be, the important fact remains that the sensitivity curve of the protanope may not be the result of the activity of a single receptor, but

could arise by the interaction of two or more receptors, and the experiments, with adaptation to red and violet, on the whole suggest that a composite pathway is the more likely.

A study of the 'intermediates' among the deuteranopic observers shows that there is a tendency for the adaptation effects of violet on violet, and of violet on red, to approximate to each other (see Fig. 2), and similarly for the effects of red adaptation on red threshold to become identical with those of red adaptation on violet threshold, but the effects of violet adaptation are always somewhat greater than those of red adaptation. This suggests that the violet light has more power of causing adaptation than the red light. This could arise if the violet-adapting light was for some reason actually more intense than the red, i.e. if the determination of its threshold were in some way inaccurate, or if the absolute foveal threshold had not been obtained owing to imperfect fixation. By repetition of the tests, however, no evidence has been obtained to suggest that this is the cause, but the possibility cannot be entirely ruled out. Secondly, it is conceivable that violet light works slightly differently from red light in producing the photochemical changes in the receptor. For example, Chase & Smith (1940) have observed that in the regeneration of visual purple after bleaching *in vitro*, the process is quicker after bleaching with blue than with yellow light. Another possibility lies in the nature of the change from observer 1 to observer 4. Observer 1 clearly has two types of receptor, while observer 4 only appears to have one. If this change takes place in stages, the proportions of the two types of receptor must change; then, if the numbers of receptors as well as their own sensitivities contribute to luminosity as they undoubtedly do for small areas in the fovea where the law 'intensity × area = constant' approximately holds, the receptor which is present in smaller numbers, if it is to give rise to the same sensation of brightness as the other receptors, must make up for this deficiency in number by being stimulated more intensely, while the other receptor, which is becoming relatively and perhaps actually more numerous, can give the same luminosity at a lower intensity. Hence the system of matching the strengths of the adapting lights by determination of the thresholds may lead to a more powerful effect of violet than of red, and a reduction of the effect of red. There is some evidence that this is in fact occurring. Observer 2, for example, was not completely colour-blind in the central fovea, but could distinguish violet from other colours. He shows approximation of the two curves expressing the effects of red adaptation at a low level, and approximation of those for the effects of violet adaptation at a higher level.

In the process of reduction of numbers of the violet receptor there must come a time when the receptor becomes ineffective and the intensity of violet becomes such that it stimulates the opposite (red) receptor to such an extent that this receptor now determines the threshold. As long as the violet receptor was functioning at all, one might expect that the subject would be capable of

distinguishing some colour with the foveal centre, but all those observers whose curves are more like that of observer 4 than those of observer 2 are in fact totally colour-blind, so this may not be the explanation of the effect, unless one assumes that for colour discrimination more differentiation between the receptors is needed than can be seen to exist in relation to brightness discrimination.

Another point of some interest in relation to these curves, and, if it can be shown to be a genuinely significant effect, a difficulty in the way of accepting the hypothesis that observers like observer 4 have only one type of receptor in the central fovea, is the observation that for some of these subjects there is evidence for an actual sensitization to red at the lower brightnesses of the red adapting light. This effect may be inherent in the experimental conditions and is more noticeable in some subjects than in others but, if it is true, it points to a difference between the receptors for red and those for violet, which is in exact opposition to the main trend of the experimental results. However, these are all matters which can only be elucidated by further research.

There is also a further observation which is perhaps inconsistent with the general ideas put forward in this paper: the inconsistency lies in the forms of the luminosity curves for the normal observer and for the deuteranope of the second type in the red end of the spectrum. If the deuteranope, type II, possessed cones only in his fovea one might expect him to be more sensitive in the red end of the spectrum than the normal person, but in fact the evidence, so far, points in the opposite direction, and normal observers seem to be the more sensitive in this region.

Although there are thus inconsistencies and difficulties in the interpretation of some of the results, yet it is clear that, allowing for the inaccuracy which seems necessarily to accompany work with small fields, the foveal centre is essentially a simpler part of the eye than the fovea as a whole and in the deuteranope, type II, it seems to reach the height of simplification and possess but one receptor path. It is difficult to harmonize these results with the ideas of multiple types of receptors for the normal eye.

SUMMARY

1. By adapting the fovea separately to lights of equal brightness from the two ends of the spectrum, it has been shown that the central fovea of the normal subject has its thresholds to red and to violet changed in a characteristic manner.

2. When red-green-blind subjects are treated in the same way they fall into the following classes:

(*a*) Protanopes, whose red threshold is raised equally by red and violet adaptation, whose violet threshold is raised equally by red and violet adaptation, but whose violet threshold is raised more by both red and violet than is the red threshold.

(*b*) Deuteranopes, type I, whose pattern of adaptation does not differ from that of the normal subject.

(*c*) Deuteranopes, type II, whose red and violet thresholds are raised to the same extent by both red and violet adaptation.

(*d*) Deuteranopes intermediate between types I and II and showing gradation towards type II, but with the effect of violet adaptation on violet threshold generally somewhat higher than in type II.

3. Each of these groups has a characteristic luminosity curve, thus:

(*a*) Protanopes. Maximum sensitivity at 540 mμ. and depressed sensitivity to red.

(*b*) Deuteranopes, type I. Maximum sensitivity at 550 mμ. and indistinguishable from normal subjects.

(*c*) Deuteranopes type II. Maximum sensitivity at 570 mμ. with depressed sensitivity in the blue and green and in the extreme red.

(*d*) Deuteranopes intermediate between types I and II. The curve lies between the normal and that of the deuteranopes, type II.

4. These results may be interpreted as follows:

(*a*) The protanope has a single pathway from his central fovea, but this pathway (*ab*) may be stimulated by two receptors (*AB*).

(*b*) The deuteranope, type II, has only one type of receptor (*B*) and one pathway (*b*) from his central fovea.

(*c*) The normal observer has both the receptors (*AB*) and pathway (*ab*) of the protanope together with the receptor (*B*) and pathway (*b*) of the deuteranope type II.

(*d*) The deuteranope, type I, has the same receptors as the normal subject, but his pathways fuse before reaching the 'colour centre'.

(*e*) The intermediate types of deuteranopes show a progressive elimination of the $AB \rightarrow ab$ system, eventually leaving only the $B \rightarrow b$ system of the deuteranope type II.

5. The luminosity curve for the foveal centre of the deuteranope, type II, probably represents the spectral sensitivity of the red receptor (*B*) of the normal eye. It is very similar in shape to that of a visual purple receptor, but its maximum is at about 570 mμ. instead of 507 mμ.

6. The manner in which macular pigment may function to modify the results, particularly in relation to protanopic vision, is discussed.

I acknowledge with gratitude the receipt of a grant from the Government Grants Committee of the Royal Society for apparatus, and from the Leverhulme Trustees for a personal grant which enabled me to carry out the work while on leave of absence from University duties.

I am also heavily indebted to all those kind-hearted subjects who have co-operated so unsparingly in the work and devoted so much time and effort towards the solution of the problems of the central fovea. They are too numerous to mention by name, and many may for obvious reasons wish to remain anonymous, but their services have been noble and I thank them most sincerely.

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POSITIONAL NYSTAGMUS OF PERIPHERAL ORIGIN

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Three types of positional nystagmus, i.e. nystagmus appearing or changing in direction in special positions of the head, may occur (Nylén, 1943; Seiferth, 1927): (1) nystagmus of constant direction appearing only in certain positions of the head; (2) nystagmus changing in direction with varying positions of the head; (3) irregular positional nystagmus. Although it is generally accepted (Lindsay, 1946) that types 2 and 3 are of central origin and that type 1 may be of peripheral or central origin, some clinical observations suggest that peripheral abnormalities may produce positional nystagmus of varying direction (type 2).

Gerlings (1948) described four cases of patients who most probably had such a nystagmus. As the origin of a symptom in a patient suffering from inflammation of the ear is never quite certain without a post-mortem examination, some observations on rabbits may be of importance in establishing the origin of positional nystagmus.

METHODS

In order to examine the results of destruction or stimulation of the saccule in rabbits the bulla of the ear was opened from the ventral side of the head (Versteegh, 1927). The lateral wall of the cochlea was opened with a small chisel or hook. The stapes was taken away but the upper margin of the oval window left intact. Of the modiolus so much was taken away that the utricular membrane was clearly visible. In some of the animals the saccular macula was destroyed by scratching it with a bent needle. In another series a small steel ball was put into the open pars inferior labyrinthi touching the intact saccule; the steel ball was kept in place by the lateral wall of the cochlea, put back into its original place. The operations were performed under paraldehyde-narcosis (1·5 c.c./kg. orally) with the aid of a binocular microscope (working distance 22 cm.). The wounds were dressed with penicillin and a small piece of gauze.

RESULTS

The saccule was destroyed in twenty experiments. In one case only was nystagmus observed.

* Director: Professor Dr A. A. J. van Egmond.

Case 1. *Rabbit* 17

Before the operation normal vestibular reactions; no spontaneous nystagmus or other abnormalities.

8. vi. Destruction of the left saccule.

9. vi. Normal behaviour; walking and sitting quite normal. Normal position: no nystagmus; right-side-down position: vertical nystagmus upwards with a clear rotatory component; left-side-down position: vertical nystagmus downwards, without rotatory component. Caloric reactions with cold and hot water normal in duration and direction. No fistula symptom. Turning reactions: duration of nystagmus after turning clockwise and anticlockwise identical for horizontal, vertical and rotatory nystagmus.

11. vi. Same symptoms. The positional nystagmus is much weaker and the rotatory component in the right-side-down position has disappeared. In the course of the next few days the positional nystagmus gradually disappears.

16. vi. Destruction of the right saccule. No positional nystagmus is produced.

A metal ball was placed in nine labyrinths; twice a positional nystagmus appeared.

Case 2. *Rabbit* 13

Before the operation normal vestibular reactions.

22. iv. Left labyrinth opened; a small steel ball (8 mg.) is put into the pars inferior, without opening the pars superior or saccule (see Fig. 1). The ball was sterilized in spirit.

23. iv. Apparently the left labyrinth is destroyed (head turned left side down, etc., all the symptoms of total labyrinth destruction).

In the normal position no nystagmus (deviation to the left); right-side-down position: no nystagmus; left-side-down position: vertical nystagmus downwards.

The animal was killed the next day. Histological examination of left labyrinth: macula sacculi intact, bleeding in pars superior.

Case 3. *Rabbit* 14

Before the operation normal vestibular reactions.

29. iv. Right labyrinth opened; a small steel ball (6 mg.) is put into the pars inferior without opening the pars superior or saccule.

30. iv. Apart from slight turning of the head towards the right (about 30°), normal behaviour. Normal position: no nystagmus; right-side-down position: vertical nystagmus downwards; left-side-down position: horizontal nystagmus towards the right.

1. vi. Head is kept in the normal position; caloric reactions with hot and cold water normal. Turning reactions normal. The positional nystagmus is weaker but shows the same pattern.

3. vi. No abnormalities are found. The animal was killed on 5 June. Histological examination shows a normal pars superior and a total absence of the saccular macula.

DISCUSSION

According to Nylén (1929) the origin of positional nystagmus is to be sought in changes of intravestibular pressure, produced by changes in position. Changes in the perilymphatic space (e.g. bleeding) might produce suitable conditions for the appearance of positional nystagmus. Centrifuging the otoliths from their maculae gave rise to positional nystagmus in several experiments (Hasegawa, 1925; Nylén, 1926). Removal of the macula sacculi did not influence positional nystagmus caused by alcohol intoxication (de Kleyn & Versteegh, 1930). Introduction of amalgam into the inferior part of the labyrinth caused positional nystagmus if the amalgam touched the utricular membrane.

Fig. 1. Röntgen film of the head of a rabbit. The steel ball is in the vestibule of the right labyrinth; the lateral wall of the cochlea is missing.

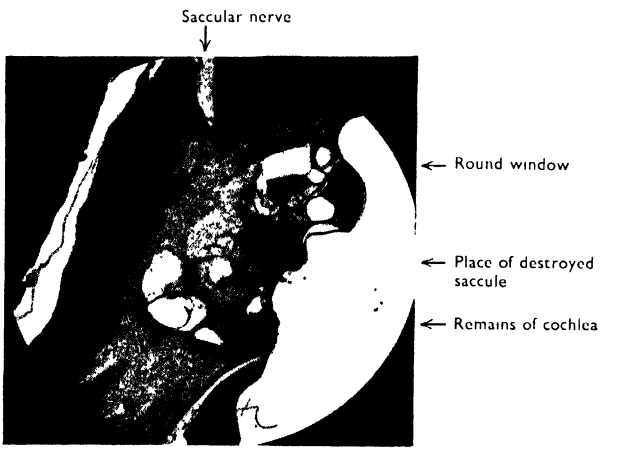

Fig. 2. Histological picture of the left labyrinth of rabbit no. 17 (horizontal section).

Fig. 3. Histological picture of the right labyrinth of rabbit no. 14 (vertical section). At the top the saccular nerve is seen. There is no trace of the saccular macula although its site was not destroyed. The circle indicates the (removed) steel ball.

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From the present experiments the following conclusions may be drawn. Positional nystagmus may be caused by damage to the peripheral labyrinth, even when the function of the semicircular canal-system seems to remain normal (functional and histological control, case 1). Positional nystagmus with changing direction may be caused by lesions of the peripheral labyrinth (cases 1 and 3). Vertical nystagmus may originate from lesions of the peripheral labyrinth (Spiegel & Scala, 1944; Rachlis, 1945).

It is not possible to draw certain conclusions about the influence of the saccule, as opening the pars inferior labyrinthi causes changes in the intra-labyrinthine pressure of the entire labyrinth. The fact that positional nystagmus appeared after destruction of the saccule (case 1) does not prove that the saccule plays no part in peripheral positional nystagmus. It may be that irritation of the macula sacculi produces it before destruction is complete. The disappearance of the positional nystagmus after some days might be due to gradual death of the cells of the saccular macula (case 3) following the initial trauma. The centrifuging experiments of Nylén (1926) might be similarly explained.

SUMMARY

1. Destruction of the saccule in rabbits gave a positional nystagmus in 1 case out of 20.

2. A small steel ball (approx. 6 mg.) placed in the vestibule of the labyrinth, beside the intact saccule, produced positional nystagmus in two cases out of nine.

3. Positional nystagmus with changing direction may be caused by damage to the pars inferior labyrinthi, even though the function of the semicircular canals appears normal.

4. Vertical (positional) nystagmus may be caused by lesions of the peripheral labyrinth.

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BICARBONATE ION AND STRIATED MUSCLE

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A slip of rat diaphragm contracts steadily for many hours when stimulated at intervals in physiological saline buffered by carbon dioxide and sodium bicarbonate ('bicarbonate-saline'). It was noticed that muscles in bicarbonate-free saline buffered by phosphates ('phosphate-saline') do not become steady but show a continuous decline in tension. The action of the CO_2-bicarbonate buffer system was investigated as follows.

METHODS

The rat diaphragm preparation with its phrenic nerve was used as described by Bulbring (1946). The right side of the diaphragm was chosen, as this contains more muscle and less tendon. For direct stimulation the muscles were denervated at least 12 days previously, about 2 cm. of the right phrenic nerve being avulsed at operation. A succession of just supramaximal single stimuli was employed from a thyratron square-wave generator giving pulses at 4 per min.; a duration of 0·25 m.sec. and voltage of 0·7–1·0 V. was used for nerve stimulation, while 1 m.sec. duration and 50–60 V. were required for direct stimulation of denervated muscle, the stimulating cathode being formed by the wire attaching the tendon to the lever. Such durations do not produce repetition. The preparation was stimulated beneath the surface of the saline.

Krebs solution or modifications were employed. This solution is similar to a serum ultrafiltrate, and its preparation is given in Table 1. In most experiments, bicarbonate-saline equili-

TABLE 1. Solutions used

	Normal Krebs solution	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)
0·9% NaCl (0·154M) (ml.)	100	396	480	402	486	486	486	486
1·3% $NaHCO_3$ (0·154M) (ml.)	21	84	—	84	—	—	—	—
1·15% KCl (0·154M) (ml.)	4	20	20	20	20	20	20	20
1·22% $CaCl_2$ (0·11M) (ml.)	3	12	12	6	6	6	6	6
3·82% $MgSO_4.7H_2O$ (0·154M) (ml.)	1	4	4	4	4	4	4	4
2·11% KH_2PO_4 (0·154M) (ml.)	1	—	—	—	—	—	—	—
$NaH_2PO_4.2H_2O$ (g.)	—	—	—	—	—	—	—	0·072
$Na_2HPO_4.12H_2O$ (g.)	—	0·220	0·220	0·220	0·220	0·165	0·055	0·055
Na_3PO_4 (g.)	—	—	—	—	—	0·025	0·075	—
H_2O (ml.)	—	4	4	4	4	4	4	4
Glucose (g.)	0·26	0·52	0·52	0·52	0·52	0·52	0·52	0·52
Gas	5% CO_2	5% CO_2	O_2	5% CO_2	O_2	O_2	O_2	O_2
pH	7·45*	7·52	7·5	7·52	7·5	*c.* 8·1	*c.* 8·8	6·4

* Lehmann, 1937 (*b*).

brated with a CO_2–O_2 mixture was exchanged for phosphate-saline through which oxygen was bubbled, without change in pH. The actual solutions used are given in Table 1. Both the buffer systems—CO_2-bicarbonate and phosphate—independently bring the pH to about 7·5; therefore by exchanging solutions (*a*) and (*b*) it is possible to remove the CO_2-bicarbonate buffer system from the external medium without change of pH and with no change in the concentration of Na_2HPO_4. The first column of the table gives the preparation of 130 ml. of normal Krebs solution: subsequent columns show the preparation of 520 ml. modified solution using the three phosphates of sodium (in place of KH_2PO_4), so that the concentration of PO_4 remains constant at 11 mg. %. The crystalline phosphate is added just before use, and the calcium solution was used fresh.

Continuous recordings of pH were made by means of a glass electrode which was present in the bath with the muscle, connected to a calibrated electrometer giving direct readings to 0·02 pH. The reference half-cell, also in the 60 c.c. bath, contained both the Ag-AgCl electrode, and the bridge solution of saturated KCl which must make a liquid junction with the saline in the bath without serious leak of potassium. A ground glass joint with a tightly fitting sleeve was eventually found satisfactory, and controls showed that with a properly fitted half-cell in the bath, the muscle contracts without change for at least 2 hr. In practice, the solution in the bath was changed about every 45 min., and recordings of the muscle made with and without the electrodes in the bath were similar. The glass electrode was first standardized against M/20 potassium hydrogen phthalate, whose pH at 38° C. was taken as 4·015 (MacInnes, Belcher & Shedlowsky, 1938).

All solutions were equilibrated at 38° C. with the appropriate gas (O_2 or CO_2–O_2 mixture) before being added to the bath. Controls showed that the short period during the change-over when the muscle was in air did not produce significant alteration. When CO_2-bicarbonate-saline was removed and phosphate-saline substituted, the latter was previously saturated with oxygen by bubbling the gas through for 1 hr., so that after the change-over the CO_2 was rapidly eliminated and the pH of the bath kept constant. Denervated diaphragms are very sensitive to temperature changes: the temperature was maintained at $38{\cdot}0 \pm 0{\cdot}2°$ C.

For routine purposes the contraction height was recorded by a simple free-weighted 'isotonic' lever writing on a very slow drum. As the overswing of such a lever might be variable, results were checked by recording tension by Sandow's (1944) piezo-electric method using a gramophone pick-up containing a Rochelle salt crystal (torsional). Single-stage amplification proved sufficient, as the latency changes were not studied. The twitch lasted some 25 m.sec. and the time-constant of the amplifier was 70 m.sec. The oscillograph deflexion is proportional to a sudden tension applied to the stylus of the pick-up, as shown by the calibration in Fig. 3. The resting tension was not measured, but arranged to give maximum response.

RESULTS

Removal of CO_2*-bicarbonate buffer.* When bicarbonate-saline with 5% CO_2 (solution *a*) is replaced by phosphate-saline with oxygen (solution *b*) the following changes occur (Fig. 1). There is an immediate initial small rise in contraction height; and this is followed after a few minutes by a slow decline. The contraction height slowly diminishes over a period of some hours; this is not due to 'fatigue', for the rate of stimulation is only 4 per min. A slow drum was used, for with a fast drum the decline may not be so apparent. When CO_2-bicarbonate-saline is restored, a small diminution is usually seen at first, followed by a rapid and dramatic recovery to a level approaching the original condition.

A similar result is obtained with denervated muscle (Fig. 2), so that the site of the action would appear to be the muscle itself and not the nerve or the region of the neuromuscular junction. The experiment was also repeated by

recording the tension with the crystal myograph (Fig. 3); the tension changes are similar to the records of the contraction height obtained by the isotonic method.

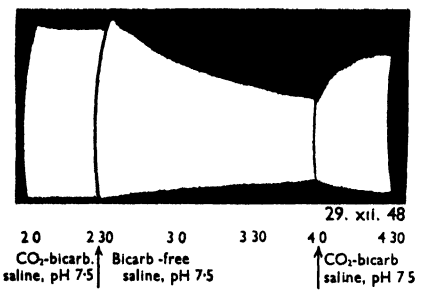


Fig. 1.

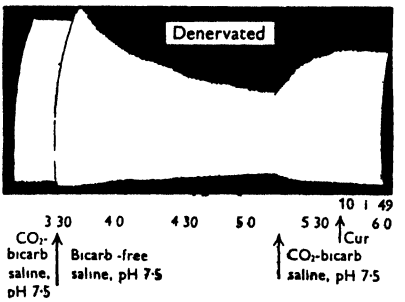


Fig. 2.

Fig. 1. Muscle stimulated via nerve. At 2.30 p.m., change from CO_2-bicarbonate-saline (solution *a*) to bicarbonate-free 'phosphate-saline' (solution *b*) at same pH. Phosphate-saline changed at 3.15 p.m. At 4.0 p.m. replace solution (*a*).

Fig. 2. As Fig. 1, using denervated muscle. At 5.40 p.m., add 200 μg. D-tubocurarine chloride to the 60 c.c. bath.

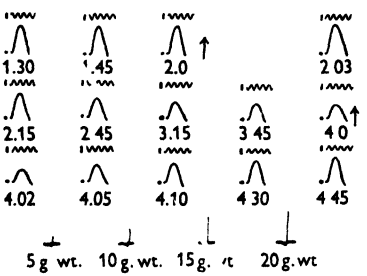


Fig. 3.

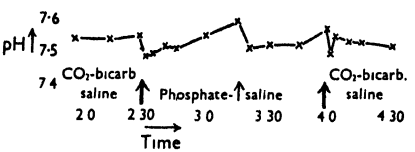


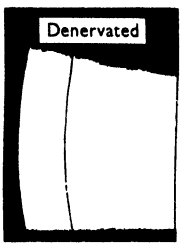
Fig. 4.

Fig. 3. As Fig. 1, recording tension. Time-marker 100/sec. At 2.01 p.m., change from solution (*a*) to solution (*b*). Phosphate-saline changed at 2.30, 3.0 and 3.30 p.m. At 4.01 p.m., restore solution (*a*). Calibration shows deflexion produced by sudden application of tensions of 5, 10, 15 and 20 g. weight.

Fig. 4. pH measurements during experiment shown in Fig. 1. There is a variation of only 0·10 pH unit throughout.

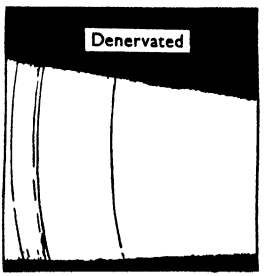
Solutions (*a*) and (*b*) have the same sodium content, osmotic pressure and ionic strength; the same amount of disodium phosphate is present in each, and the pH remains substantially constant; they differ only in that solution (*a*) is equilibrated with 5 % carbon dioxide and contains 148 mg. % sodium bicarbonate (25 mM) in place of sodium chloride. Hence the changes in Figs. 1 and 2 are to be attributed to the action of carbon dioxide and the bicarbonate ion. That the pH is essentially constant is seen from Fig. 4, which gives the pH readings during the experiment of Fig. 1. There was a variation in this case of 0·10 pH

unit during the whole experiment. This is rather better than usual, but the variation can usually be kept within 0·15 pH unit without difficulty. Such variations are to be expected, since the phosphate-saline contains only 11 mg. % PO_4 —approximately the concentration found in serum—and is poorly buffered; it



4 30 4 0
Phosphate-saline + O_2, pH 7·4 ↑ Phosphate-saline + O_2, pH 8·1

Fig. 5.

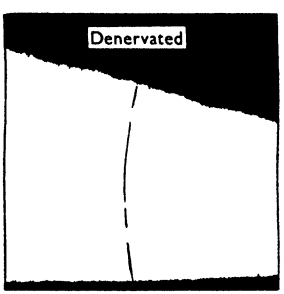


4 0 4.30 5.0
Phosphate-saline + O_2, pH 7·5 ↑ Phosphate-saline + O_2, pH 8·8

Fig. 6.

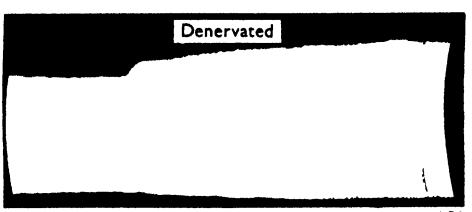
Fig. 5. Denervated muscle in phosphate-saline; contractions slowly diminishing. At 4.30 p.m. change from pH 7·5 to 8·1 (solution *d* to *e*).

Fig. 6. As Fig. 5. At 4.30 p.m., change from pH 7·5 to 8·8 (solution *d* to solution *f*). Faint precipitate.



2 30 3.0 3 30
Phosphate-saline + O_2, pH 7·5 ↑ Phosphate-saline + O_2, pH 6·4

Fig. 7.



1 30 2 0 2 30 3 0 3 30 4.0 4 30
5% CO_2 + 95% O_2 ↑ 100% O_2

Fig. 8.

Fig. 7. As Fig. 5. At 3.0 p.m., change from pH 7·5 to 6·4 (solution *d* to solution *g*).

Fig. 8. Denervated muscle in CO_2-bicarbonate saline (solution *c*) at pH 7·5. At 2.0 p.m., give 100% O_2 instead of 5% CO_2-95% O_2 mixture; pH 8·8.

is, however, possible by this means to maintain the pH reasonably constant while avoiding abnormal concentrations of phosphate or the use of unphysiological buffers.

pH *changes in phosphate-saline.* It has been shown above that after a muscle has been depressed by a phosphate-saline solution, a remarkable recovery

occurs when this solution is replaced by saline containing the CO_2-bicarbonate buffer system. Merely changing the pH of the phosphate-saline produces no such recovery. Fig. 5 shows an experiment on denervated muscle using half calcium solution to avoid precipitation of calcium phosphate. A rise from pH 7·5 to 8·1, produced by replacing part of the Na_2HPO_4 by an equivalent amount of Na_3PO_4, produced no recovery (Fig. 6); in Fig. 7, the muscle shows no recovery even at pH 8·7–8·9; a small precipitate of calcium phosphate is apt to form at high pH, but has no apparent effect on the muscle. A fall of pH from 7·5 to 6·4 likewise produced no marked change (Fig. 6). In general, the slow depression occurring in phosphate-saline proceeds somewhat more quickly in an acid medium and with a high calcium concentration.

Effect of carbon dioxide changes alone. The prolonged depression which occurs in phosphate-saline cannot be attributed to lack of free carbon dioxide. Fig. 8 shows that absence of CO_2 gas produces a permanent increase in the contraction height of denervated muscle. Turning again to Figs. 1 and 2, it is seen that the effect of removal both of CO_2 and of bicarbonate produces an initial small rapid rise before the familiar slow depression. It appears that this initial rise is produced by the fall in CO_2 tension; the slow depression that later supervenes is associated with absence of bicarbonate. Similarly, when the phosphate-saline is removed and CO_2-bicarbonate restored, an initial small depression precedes the recovery. Carbon dioxide is known to depress muscle (Haywood, 1927).

Action of bicarbonate alone. After a muscle has been depressed in phosphate-saline, recovery will occur in bicarbonate-saline without CO_2 gas (Fig. 9). In fact, without CO_2 the recovery is better. The pH of the bath rises to 8·8, but from Fig. 6 the recovery of the muscle cannot be attributed to the pH change of the external fluid. Hence it may be said that removal of the bicarbonate ion produces a slow diminution in contraction height (and tension), and replacement of the bicarbonate ion produces recovery.

A note on solutions

Krebs solution provides a convenient approximation to a serum ultrafiltrate. Devised originally for biochemical use, it has been used in mammalian

TABLE 2

	Krebs solution	Mammalian serum	Tyrode solution
Na	327	320	342
K	23	22	10·5
Ca	?5	?5	7
Mg	2·9	2·5	2·6
Cl	454	370	515
HCO_3	148	148	72
PO_4	11	10	4
SO_4	11·4	11	—
pH	7·45	7·4	7·15

Concentrations in mg. %, the first two columns slightly modified from Krebs & Henseleit (1932); the saline solutions are equilibrated with 5% CO_2.

nerve physiology (Lehmann 1937*a*; Lorente de Nó, 1947). Table 2 gives the composition, together with that of Tyrode solution. The calcium ion concentration of Krebs solution—10 mg. %—may well be too high, as probably only a fraction of plasma calcium is ionized; half-calcium saline containing 5 mg. calcium % was used in the experiments shown in Figs. 5–9. The magnesium sulphate and potassium phosphate may be omitted for routine use.

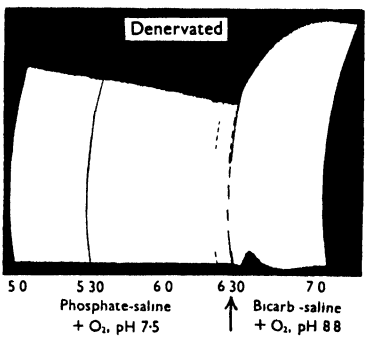


Fig. 9.

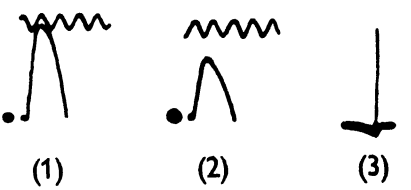


Fig. 10.

Fig. 9. Denervated muscle in phosphate-saline at pH 7·5 (solution *d*). At 6.30 p.m., change to bicarbonate-saline (solution *c*) without adding CO_2; pH 8·8.

Fig. 10. Tension of muscle stimulated via nerve in (1) normal Krebs solution and (2) after 1½ hr. in Tyrode solution, (3) shows calibration with 20 g. weight. Time-marker 100/sec.

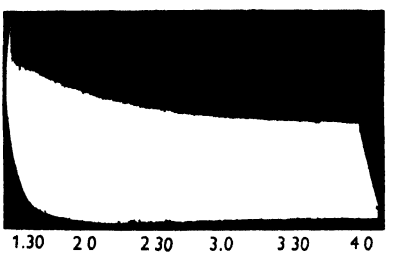


Fig. 11. Initial changes when muscle is set up in Tyrode's solution with 5% CO_2, recorded by free-weighted lever. The base-line at first falls. The contraction height also diminishes; in Krebs solution the contraction height initially increases. Temp. 38° C.

A muscle in Tyrode solution equilibrated with a mixture of 5% CO_2 and 95% O_2 gives about 60% of the tension produced in Krebs solution (Fig. 10); this is chiefly due to the low potassium content of Tyrode solution, and partly to its rather low bicarbonate concentration. A freshly removed muscle put into Krebs solution commonly increases somewhat in tension and in contraction height during the first hour as it recovers from the asphyxia and trauma of dissection; but a muscle put initially into Tyrode solution (with 5% CO_2) decreases in contraction height before becoming steady (Fig. 11). Tyrode (1910) originally used oxygen without carbon dioxide, and some

attempt has been made to employ this solution in the same way for isolated mammalian nerve-muscle preparations (Chou, 1947). The pH is very variable and may reach 9·0 with vigorous oxygenation. Preparations stimulated via the nerve in such a medium begin a slow decrease in tension after a few hours; this is not due to 'fatigue', and may be associated with the slow irreversible depolarization undergone by mammalian nerves deprived of carbon dioxide (Lorente de Nó, 1947).

DISCUSSION

The experiments which have been described appear to indicate that the bicarbonate ion is necessary for normal function of the rat diaphragm, quite apart from buffering of the external medium. A similar investigation was carried out many years ago by Rona & Neukirch (1912) on rabbit's intestine. They found that spontaneous contractions in bicarbonate-free saline, buffered by glycine, were poor or absent; they were much impressed by the effect of bicarbonate in producing contractions, and showed that this could not be explained by the pH of the medium. Berg (1911) fatigued a frog's sartorius muscle in bicarbonate-free saline and found that bicarbonate assists recovery.

The effect of bicarbonate may perhaps be attributed not to the external pH but to the maintenance of internal pH. On this view, tissues in bicarbonate-free solutions lose bicarbonate and the internal pH falls. The muscle membrane is believed to be permeable to the bicarbonate ion (Boyle & Conway, 1941). This may help to explain the shape of Figs. 1 and 2, which show a slow decline and a much more rapid recovery. The bicarbonate ion concentration of the fibre water is probably small—less than 1 mM for rabbit's leg muscle (Conway & Fearon, 1944). Hence in a bicarbonate-free medium, there is a rather small concentration difference for ionic exchanges. When bicarbonate is again restored, in a concentration of about 150 mg. % (25 mM), the relatively large concentration difference enables the ionic exchanges to reach equilibrium much more rapidly.

Much recent work has emphasized the importance of the CO_2-bicarbonate system in metabolism. Warren (1944) found that the respiration of suspensions of rabbit bone-marrow was 40 % higher in bicarbonate-saline than in phosphate-saline. Pigeon liver and other tissues possess the well-known ability to utilize carbon dioxide in reactions leading to metabolic synthesis (Krebs & Eggleston, 1940), but this apparently does not apply to pigeon muscle (Evans & Slotin, 1941). That bicarbonate and carbonic anhydrase accelerate certain ionic exchanges across the erythrocyte membrane has been shown by Jacobs & Stewart (1942) and by Keilin & Mann (1941); but according to Van Goor (1940), muscles possess very little carbonic anhydrase.

SUMMARY

1. A study has been made of the action of the carbon dioxide-bicarbonate buffer system on the tension and contraction height of the isolated rat diaphragm muscle, both normal and denervated, using ionic concentrations similar to those of plasma.

2. Presence of bicarbonate in the external fluid is necessary for the maintenance of normal twitch tension. A slow decline in tension occurs in the absence of bicarbonate; this is independent of pH changes of the environment, and is largely reversible.

It is a pleasure to acknowledge the help received from Prof. R. J. S. McDowall and Dr I. Hajdu.

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EXCRETION OF PARA-AMINOHIPPURATE BY THE KIDNEY OF THE CAT

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The kidney of the cat has been shown to behave differently from that of man or the dog in its manner of excreting thiosulphate (Eggleton & Habib, 1949), and an investigation has therefore been made to determine the manner in which para-aminohippurate (PAH) is excreted. In both man and dog, this substance is actively secreted by the tubule cells (Smith, Finkelstein, Aliminosa, Crawford & Graber, 1945); at low concentrations in the plasma, it is almost completely removed from the blood during its passage through the kidney, so that its clearance provides a measure of the minimal renal plasma flow, and at higher concentrations, a maximal rate of secretion is rapidly attained, this value providing a measure of tubular function (*Tm*).

METHODS

The general technique adopted has been already described (Eggleton & Habib, 1949), all experiments being performed under nembutal anaesthesia. In general, an initial priming dose of PAH was given, followed by a steady infusion of the substance, of such strength as to maintain either a steady concentration of PAH in the plasma or a steadily increasing concentration. In several instances, the infusion was stopped half-way through the experiment, and subsequent determination of clearance made as the plasma PAH concentration decreased. Creatinine clearance was used as a measure of glomerular filtration rate (G.F.R.) for reasons already given in the previous publication; the creatinine was given either in a single dose intravenously or in one or more doses subcutaneously.

Creatinine was determined colorimetrically as the alkaline picrate. PAH was determined by the method of Smith *et al.* (1945), with the slight modification already described (Eggleton & Habib, 1949). The blood samples (arterial) were centrifuged immediately as advised by Smith *et al.* (1945), although analysis of the cells showed them to contain little or no PAH. The small amount apparently contained could be accounted for if they had been contaminated with 6–8% of plasma in place of the 5% postulated by Smith *et al.* The technique adopted by those authors to test for the presence of any PAH in conjugated form was also used, with both plasma and urine. In the latter, identical values of PAH concentration were obtained before and after hydrolysis: and in plasma, hydrolysis resulted in a slight fall in concentration. Thus, there is no indication of any conjugation product of PAH.

RESULTS

Renal plasma flow

At low concentrations of PAH in the plasma, the clearance of this substance was several-fold that of creatinine and uninfluenced by changes in concentration, as can be seen from the results of parts of two experiments presented in Table 1. It would seem, therefore, that in the cat, as in man and the dog, this clearance provides a measure of minimal renal plasma flow. The average filtration fraction of twenty-one observations in seven animals was 0·305 ± 0·012, individual values varying from 0·23 to 0·46. It will be noted that, of the two examples given in Table 1, in one the plasma PAH concentration lies between

TABLE 1. Renal plasma flow and filtration fraction in the cat

Sample (min.)	Urine flow (c.c./min.)	PAH concentration: Plasma (mg./100 c.c.)	PAH concentration: Urine (mg./100 c.c.)	PAH clearance (renal plasma flow) (c.c./min.)	Creatinine clearance (c.c./min.)	Creatinine/PAH clearance ratio (filtration fraction)
			Cat, 4·5 kg.			
15	1·48	4·85	177	54	17·8	0·33
15	1·45	4·5	180	58	17·4	0·30
15	1·48	4·4	182	61	17·1	0·28
15	1·49	4·4	190	64·5	18·0	0·28
			Cat, 3·5 kg.			
7	0·64	1·85	165	57	19·5	0·34
7	0·63	1·63	148	57	19·8	0·345
8	0·61	1·45	148	62	20·7	0·33
9	0·535	1·3	155	63·5	19·6	0·31

4 and 5 mg./100 c.c. It is customary in man and dog to maintain a concentration lower than this but, in the cat, the PAH clearance appears practically unchanged up to a concentration where the maximal rate of secretion is reached. A slight fall in clearance with rising concentration could be observed in the experiments in which more than one value was obtained below 10 mg. PAH/100 c.c. plasma, but this was negligible in relation to the wide individual differences found in different cats. Thus, a PAH/creatinine clearance ratio of 4·15 was observed at a concentration of 7·3 mg./100 c.c., while the lowest ratio encountered, 2·17, occurred at a concentration of only 0·57 mg. /100 c.c.

Tm_{PAH}

With increasing concentration above 10 mg./100 c.c. plasma, the PAH/creatinine clearance ratio fell, as may be seen from the results of a typical experiment shown in Fig. 1. The depressant action of PAH on creatinine clearance is also demonstrated in this figure. It is of the same order as that observed in the inulin clearance in man (Crawford, 1948), and has been attributed to circulatory upset affecting the renal arterioles and so reducing glomerular capillary pressure.

If the amount of PAH actively secreted by the tubule cells (amount excreted – amount filtered) be plotted against concentration in the plasma, the results shown in Fig. 2 are obtained. The amount secreted rises sharply with increasing concentration to *c.* 10 mg./100 c.c. plasma, remaining approximately constant thereafter at a value of 20–30 mg./100 c.c. G.F.R./min. This is smaller than that observed in the dog (Bing, 1943) and about half that

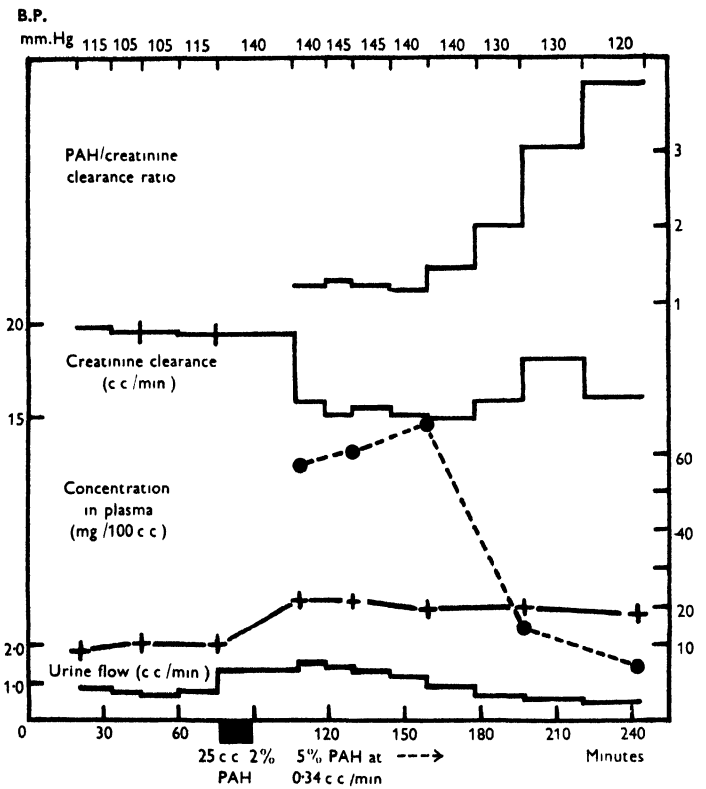

Fig. 1. Showing (*a*) the rising PAH clearance with fall in plasma PAH concentration, (*b*) the depressant action of PAH on creatinine clearance. Cat, 3·5 kg., 0·5 g. creatinine was given subcutaneously at times 0 and 78 min. and 0·3 g. at 160 min. +———+, creatinine; ●-----●, PAH.

in man (Chasis, Redish, Goldring, Ranges & Smith, 1945). The results have been corrected, since it is accepted that PAH is not freely filterable, the fraction varying from 0·87 to 0·92 in the dog and having an average value of 0·83 in man (Smith *et al.* 1945). In four cats in which the G.F.R. lay between 15 and 25 c.c./min. and the plasma PAH concentration between 10·2 and 56·3 mg./100 c.c., the amount of PAH filtered (if freely filterable) plotted against the amount excreted is shown in Fig. 3. The line through the experimental points, fitted by the method of least squares, has a slope of 0·91,

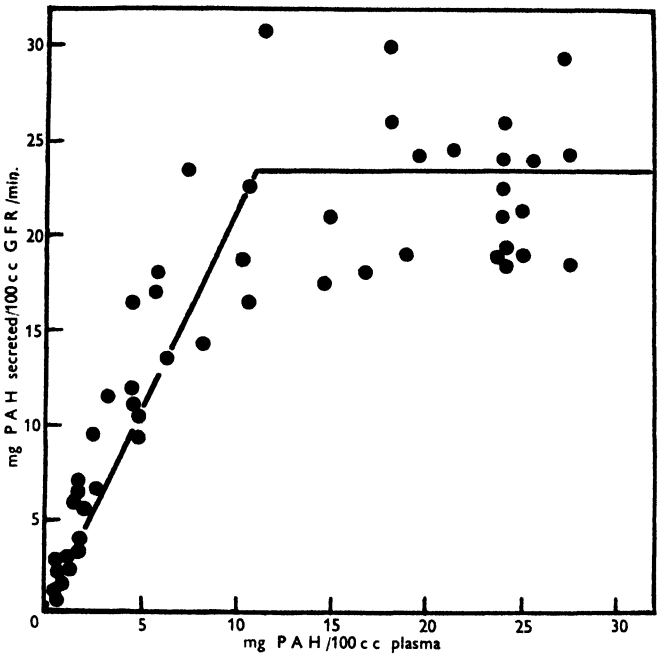

Fig. 2. Showing the rise in amount of PAH secreted with increasing plasma PAH concentration to a maximal value (*Tm*) of 20–30 mg./100 c.c. G.F.R./min. at *c.* 10 mg. PAH/100 c.c. plasma.

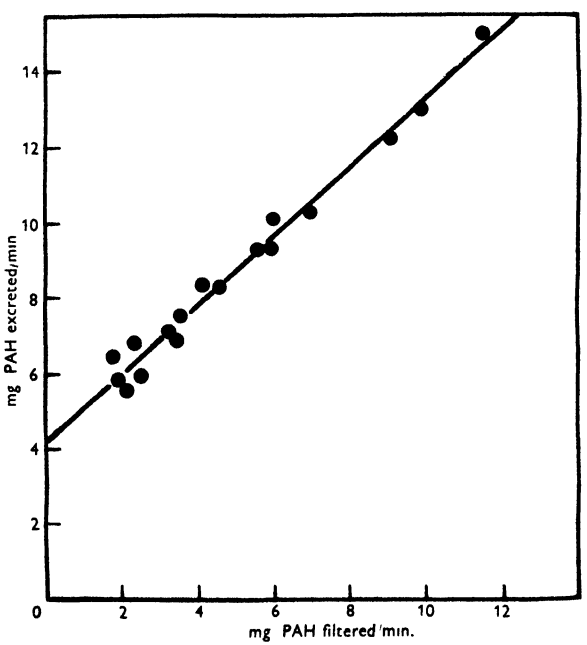

Fig. 3. Data from four cats with an average creatinine clearance of 18·3 c.c./min. The slope of the line 0·91 indicates the fraction of PAH which is filterable.

indicating a filterable fraction of the same order as that found in the dog. The point at which the line cuts the ordinate gives a value for the maximum secretion rate of the tubule cells—Tm_{PAH}—which, in this group of animals, averages 4·2 mg./min. or 23 mg./100 c.c. G.F.R./min. This correction factor of 0·91 has been applied in the calculation of the amount of PAH filtered in all experiments subsequently quoted.

Reabsorption of PAH

It will be noted that the range of PAH concentration in the plasma covered in Fig. 2 is only 0–30 mg./100 c.c. At concentrations greater than this, Tm_{PAH} was found to decrease again, the magnitude of change varying widely in different animals. Details of one such experiment are given in Table 2 and the results of two others depicted in Fig. 4. In the upper curve, apparent depression of secretion was already obvious at 30–40 mg. PAH/100 c.c. plasma. In the lower, a greater depression was apparent at 80–90 mg./100 c.c. and the amount secreted was later reduced to only 2 mg./100 c.c. G.F.R./min. In both cases full recovery occurred at a concentration which, on the rising plasma PAH curve, had proved depressant. This effect is similar to the so-called 'hysteresis' effect observed by Shannon & Troast (quoted by Goldring, Chasis, Ranges & Smith, 1940) with glucose and has, as yet, no adequate explanation. The failure of full depressant action at the highest concentrations, to be noted in all three experiments, is associated with higher rates of urine flow, which is to some extent responsible for the phenomenon. The matter has not been investigated in detail but in numerous experiments, when change in rate of flow appeared to be the only variable factor, the amount of PAH apparently secreted increased with increasing rate of flow and vice versa, unless sodium sulphate were used as the diuretic; a high concentration of this substance had the opposite effect, decreasing the amount of PAH apparently secreted in spite of increased rate of flow. Five further experiments similar to those quoted above were performed, all yielding similar results. In one of these, there was apparent reabsorption of PAH, as in that quoted in Table 2, but of far greater magnitude. At a plasma PAH concentration of 116 mg./100 c.c. the amount excreted was 15 mg./100 c.c. G.F.R./min. less than that filtered. In a further five experiments in which a concentration greater than 35 mg./100 c.c. plasma was maintained throughout the experiment, Tm_{PAH} was consistently less than 20 mg./100 c.c. G.F.R./min. and again, in one of these, apparent reabsorption occurred; at 90–100 mg./100 c.c. plasma, the amount excreted was less (2–9 mg./100 c.c. G.F.R./min.) than that filtered.

Another factor found to depress the apparent secretion of PAH is a high concentration of creatinine. The effect is most pronounced if no creatinine has previously been given, an infusion of the substance causing a profound fall in PAH clearance. In one experiment, this occurred at a concentration of

30–40 mg. PAH/100 c.c. plasma. In a second experiment of the same type, but with a low plasma PAH concentration (1–2 mg./100 c.c.), a similar depression occurred during the creatinine infusion, but was followed by immediate

TABLE 2. Showing the depression of Tm_{PAH} with increasing PAH concentration in the plasma (cat, 4·5 kg.)

Time (min.)	Creatinine clearance (c.c./min.)	Plasma PAH (mg./100 c.c.)	PAH (mg./min.) Filtered (corr.)	Excreted	Secreted	Secreted/100 c.c. G.F.R.	Urine flow (c.c./min.)
25 c.c. 2% PAH intravenously, followed by 5% PAH at 0·8 c.c./min. beginning at 0 min.							
11–12			1 g. creatinine subcutaneously				
20–32	10·9	121·5	12·0	14·2	2·2	20·0	0·71
32–44	13·1	140 5	16·75	18·3	1·55	11·9	0·88
44–59	12·8	158·5	18·5	18·1	−0·4	−3·0	1·02
59–74	11·85	175	18·9	19·05	0·15	1·2	1·16
			PAH infusion stopped				
74–89	11·9	152·5	16·5	18·65	2·15	18·0	1·05
89–105	11·9	104	11·25	14·8	3·55	30·0	1·01
105–120	11·0	78·5	7·85	11·15	3·3	30·0	0·94

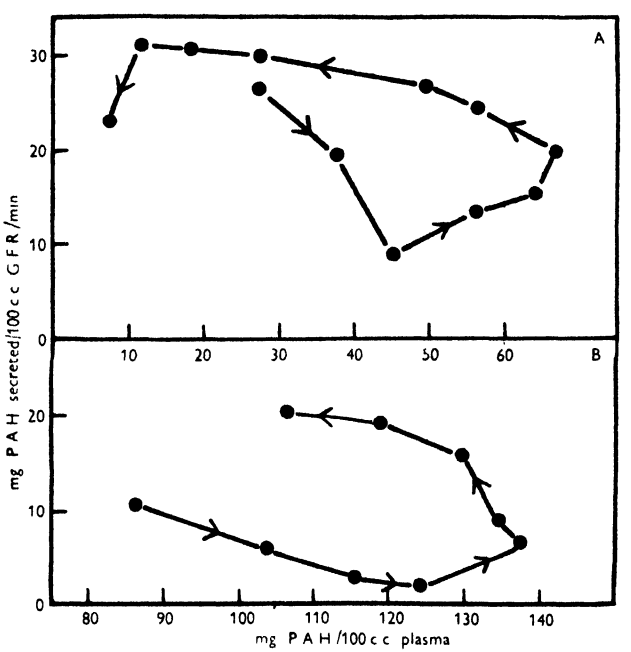

Fig. 4. Showing the reduction of Tm_{PAH} with increasing PAH concentration in the plasma and subsequent recovery with decreasing concentration in two separate experiments.

recovery. Such experiments are, however, open to criticism on the ground that the effect might be due to changes in G.F.R. Three experiments were made, therefore, in which a low concentration of creatinine was maintained in the

early part of the experiment by subcutaneous injection, thus allowing calculation of Tm_{PAH} throughout. The results of one are shown in Fig. 5, and indicate clearly that change in filtration rate is not responsible for the depression of Tm. The greatest depression, as in all these experiments, occurred at the time of highest plasma creatinine concentration, but was still obvious as the concentration fell from 107 to 50 mg./100 c.c. In this experiment, the plasma

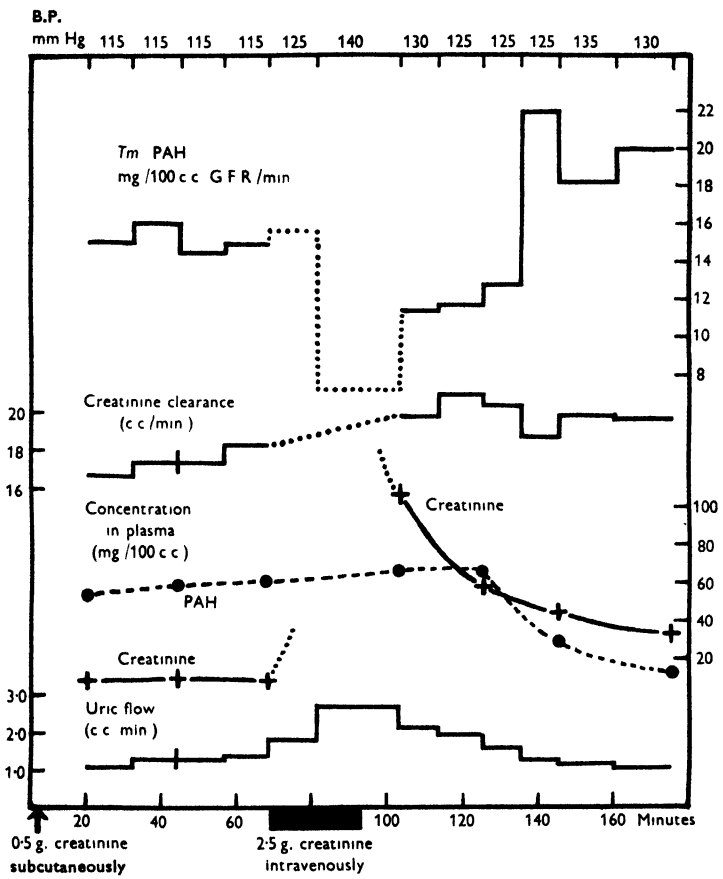

Fig. 5. Showing the depressant action of a high concentration of creatinine on PAH clearance. Cat, 4·2 kg. 5 % PAH infused from 0 to 125 min. at 0·34 c.c./min., preceded by 25 c.c. 2·5 % PAH at −11 to 0 min. +———+, creatinine; ●-----●, PAH.

PAH concentration was such that Tm_{PAH} was already partially depressed, the value rising to normal directly the PAH infusion was stopped. In a second experiment of the same nature, well-marked depression was noted only during the creatinine infusion, but in a third, an initial Tm_{PAH} of 16 mg./100 c.c. G.F.R./min. fell to 3 mg. during the infusion and recovered to 10–13 mg. as the plasma creatinine concentration fell from 122 to 55 mg./100 c.c. in the following

45 min. Full recovery (to 16 mg.) occurred when the concentration fell to 40 mg./100 c.c., the plasma PAH remaining throughout between 27 and 30 mg./100 c.c. plasma.

DISCUSSION

The general concept of assessment of renal tubular function by measurement of Tm_{PAH} or Tm of other substances secreted has proved a fruitful one and will, no doubt, continue to be so. Yet the results now presented suggest that the measurement should be regarded as empirical only, rather than as yielding information regarding the actual mode of excretion of these substances. For clinical use, the general technique for determining Tm_{PAH} is prescribed in considerable detail, so that measurements are always made within a fairly restricted range of PAH concentration in the plasma and the results obtained are, therefore, likely to yield comparable indices of tubular function in different kidneys. Before such a Tm value can be accepted as an absolute measure of tubular secretory power, however, it would seem expedient to investigate whether the kidneys of other species, including man, show the same anomalous behaviour as does that of the cat when the experimental conditions are deliberately varied.

The depressant effect of high PAH concentration on its apparent secretion is open to two interpretations. It might be due to a combination of normal secretion and back diffusion, the latter process varying with the concentration gradient from tubule to plasma, or it might be a toxic action on the tubule cells, reducing their secretory power and permitting back diffusion not normally occurring. The fact that, on more than one occasion, the amount of PAH appearing in the urine was less than that filtered, can only be explained by some process of back diffusion. The absolute concentration at which this process becomes obvious varies widely from one animal to another; apart from the three experiments in which it occurred, in three others an apparent secretion rate as low as 2–5 mg./100 c.c. G.F.R./min. was attained at a concentration of 124, 90 and 50 mg./100 c.c. plasma respectively. The variation in Tm_{PAH} with rate of urine flow is also satisfactorily explained on this hypothesis. Whether the back diffusion of PAH is due to the physico-chemical properties of the substance, as in the case of urea, or whether it is due to a toxic action, rendering the tubules 'leaky', remains an open question at the moment. If the latter interpretation were correct, it might serve to explain the depressant action of PAH on apparent G.F.R. which has hitherto been attributed to changes in the renal circulation. In this event, a similar degree of toxicity must be ascribed to several other substances, for Rennick, Moe, Lyons, Hoobler & Neligh (1947) have recently reported a similar behaviour of the tetraethylammonium ion in the dog and Barclay, Cooke & de Muralt (1949) of diodone in man.

The presence of this double process, secretion and back diffusion, renders impossible an accurate assessment of the relation between the amount of PAH

secreted and its concentration in the plasma. Thus the two straight lines drawn in Fig. 2 should probably be replaced by a smooth curve, which rises rapidly, reaches a flat peak and then slowly declines. Such a curve would indeed fit the experimental points in the lower range of concentrations more closely than does the straight line. If the same line of argument be applied to Fig. 3, the meaning to be attached to the correction factor of 0·91 becomes extremely dubious. In this connexion, it is of interest that Smith *et al.* (1945) could obtain no evidence that PAH is not freely filterable through a collodion membrane, as had been demonstrated in the case of diodone.

The effect of creatinine in depressing the secretion of PAH is not easy to explain. That the creatinine clearance is independent of large changes in concentration in the plasma renders unlikely the possibility that creatinine is actively secreted or reabsorbed by the tubule cells. One might tentatively postulate an attraction for creatinine by the mechanism responsible for secretion of PAH, although the former is not passed on, either into tubule or plasma. A similar type of reaction is well known to the biochemists in the form of 'enzyme inhibitors'; these are substances which interfere with certain enzyme reactions without themselves being affected. In general, however, such inhibitors are chemically akin to the substances whose transformation they prevent: for example, malonic inhibition of succinic dehydrogenase. The only similarity in the structure of PAH and creatinine lies in the glycine radicle, but whether this is of significance or not cannot, at present, be judged. Until further information is available it would seem 'safest to say that there may be several varieties of competition between renal mechanisms and that each requires further elucidation' (Pitts, 1946).

SUMMARY

1. With a low concentration in the plasma, PAH clearance is independent of change in concentration and may be used, therefore, as a measure of minimal renal plasma flow in the cat, as in dog and man (Table 1).

2. With increasing concentration, PAH clearance falls, the amount secreted rising to a maximum value (Tm) of 20–30 mg./100 c.c. G.F.R./min. at *c.* 10 mg. PAH/100 c.c. plasma (Figs. 1 and 2).

3. At concentrations greater than 30 mg. PAH/100 c.c. plasma, Tm_{PAH} apparently diminishes again, and at high concentrations the amount excreted may be less than that filtered (Table 2, Fig. 4). It is concluded that PAH is not only actively secreted but also passively reabsorbed.

4. Tm_{PAH} is depressed by a high concentration of creatinine (Fig. 5).

Our warm thanks are due to Dr K. H. Beyer for a supply of PAH from Messrs Sharp and Dohme Inc. when it was unavailable elsewhere.

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WATER, CHLORIDE AND POTASSIUM EXCHANGES IN ISOLATED BLOOD-PERFUSED DOG LUNGS

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The work described in this paper is the continuation of an investigation begun by Daly, Eggleton, Elsden & Hebb (1946) into the blood changes associated with the occurrence of oedema in isolated perfused dog lungs; and to what extent such changes may be used to distinguish oedema which is of unknown or 'spontaneous' origin from that which is due to phosgene. Most of the changes then found to be associated with oedema could be ascribed solely to haemoconcentration; and of all which had been investigated the plasma water and Cl values seemed the most useful in determining the onset of oedema from whatever cause. There was no evidence, however, that phosgene produced any specific changes in the blood.

From subsequent observations it appeared likely that the blood and plasma K concentration might be specifically affected by phosgene. Changes in potassium concentration in the perfusate in normal and phosgene-poisoned isolated dog lungs have been investigated and, in addition, a further analysis has been made of the alterations in plasma volume and Cl which are associated with oedema.

METHODS

The experiments were performed on dog lungs, isolated and perfused with heparinized blood at constant volume inflow using negative pressure ventilation (extra-pulmonary pressure varying from −0·4 to *c.* −9 cm. water at a rate of 12½/min.). Two independent preparations were obtained in each experiment by perfusing the right and left lungs separately and in parallel (Daly, Hebb & Petrovskaia, 1941; Hebb & Nimmo-Smith, 1946).

One preparation was treated with phosgene some 1–2 hr. after beginning perfusion; the other was used as a control. In some experiments a cross-circulation was established between the test and control lungs for limited periods before and/or during gassing.

The pulmonary arterial pressure and tidal air were recorded continuously by kymograph, using recorders of the type described by Daly (1938). The venous reservoir volume of each system was noted at 10 or 15 min. intervals. Each reservoir was calibrated and readings were made directly (accuracy ±3 ml.).

Alterations in the circulating volume of blood due to extravascular losses of water by evaporation, to lung blood volume changes, and to extravascular tissue uptake of fluid, were calculated by methods already described (Daly *et al.* 1946). Methods for blood analyses were those used earlier (Hebb & Nimmo-Smith, 1946).

Administration of phosgene. Each lung was connected by its bronchial cannula and communicating tubing (wide-bore glass and rubber tubing) to a small spirometer (210 ml. capacity), the whole system being closed and having a capacity of 300–400 ml.

Gas was injected into the airway through a glass capillary tube let into the bronchial cannula near its opening into the lung. The other end of the capillary tube was on the outside of the respiratory chamber and was connected to a 3-way glass tap which in turn communicated with a 1 ml. all-glass syringe containing the gas. A second 20 ml. syringe was attached to the third outlet of the tap, and this was used as a reservoir from which the smaller syringe could be recharged when required. (See Fig. 1.)

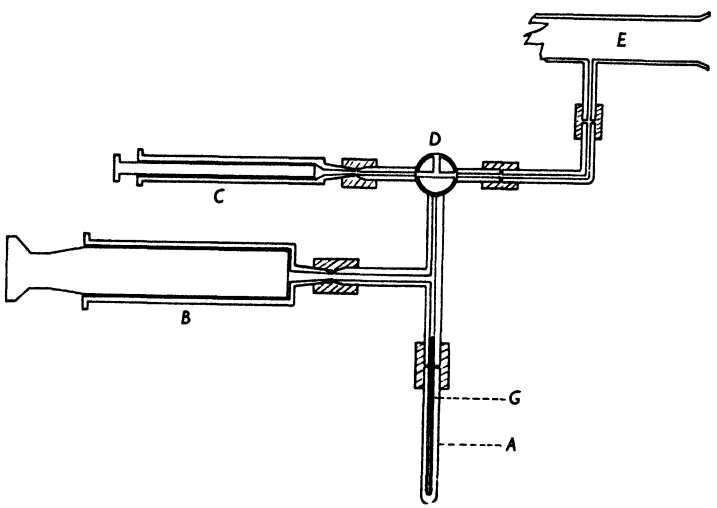

Fig. 1. Apparatus for administration of phosgene. Phosgene is admitted to the bronchial cannula (*E*), which is of wide-bore glass tubing, through the capillary glass inlet connected to the 3-way tap *D*. The small syringe *C* is filled from the larger one *B* as required and this is itself filled when the capsule of liquid phosgene *G* is broken by leverage of *A*, a sealed glass capillary tube just wide enough to admit the capsule. Except for the rubber tubing covering the glass-to-glass connexions, the apparatus is made entirely of glass. When low concentrations of gas are required the syringe *B* is partially filled with air before the capsule is exploded.

The procedure adopted was as follows. A small volume (0·05–0·2 ml.) of pure gas, or of gas mixed with air in known proportions, was injected into the airway during the short pause preceding inspiration so that the succeeding flow of air would wash it into the lungs. The injections were repeated until the required dose of gas had been given, the syringe being re-charged when necessary.

Each injection of gas was necessarily diluted by the volume of air entering the lungs at the succeeding inspiration, but this volume was measured on the spirometer tracing, and therefore the concentration as well as the total dose inhaled could be calculated. Owing to the powerful bronchoconstrictor action of phosgene, the tidal air volume was reduced during the inhalation so that the gas entered the lungs at successively higher concentrations. In some cases the increase was 4- or 5-fold with the tidal air being reduced to as little as 20% of its initial value. Since our object was to ensure that a given quantity of gas was inhaled the rise in concentration that occurred could not be avoided without prolonging the gassing over too long a period to be practicable.

In the majority of experiments the doses employed were between 6 and 20 mg. given at initial concentrations of 0·6 to 2 mg./l. of air (calculated by reference to tidal air volume). Doses of 44 and 54 mg. were tested in two experiments. These were given in concentrations of 10 mg./l. of air.

RESULTS

Conditions for the assessment of experimental oedema. In some of the early experiments carried out according to techniques similar to those used by Daly *et al.* (1946) we found, as had they, that water losses from the system occurred in the respiratory chamber as the result of evaporation from the visceral pleura of the lungs and exposed surfaces of blood. Such losses were sufficiently large to produce a haemoconcentration which masked to some extent the haemoconcentration associated with pulmonary oedema.

In later experiments, however, the modified method already described (Hebb & Nimmo-Smith, 1946) was used and such water losses were then prevented or were too small to alter the haemoglobin concentration significantly. Under these conditions the rate at which oedema developed in a given lung was followed by means of measurements of the haemoglobin (expressed as haem iron) in blood samples taken during the course of perfusion. The total volume of water lost from the blood (with or without other plasma constituents) could be calculated with an error of about $\pm 2\%$. Since the volume of blood in relation to 1 g. of lung tissue was 5–8 ml. this error when expressed in terms of the percentage change in wet weight of tissue was between 10 and 16%. In practice it was found that when the water uptake of the tissue was estimated to be 10% or more of its initial weight the presence of alveolar exudate was always demonstrable histologically.

With the modified technique the later preparations showed a general improvement in that spontaneous oedema was less frequent during the first 4–6 hr. of perfusion. Longer experimental periods were often required, however, and the occurrence of oedema in the control lungs remained a troublesome feature of the experiments. Thus out of twenty-one experiments there were only nine in which no significant extravascular loss of fluid in the controls occurred (4–9 hr. of perfusion). In the others, losses equivalent to an increase in wet weight of tissue of 0·2–1·5 g./g. tissue were found.

The effect of gassing in producing oedema was therefore only clearly evident in the nine experiments in which the controls remained in good condition. In these the gassed lungs showed an increase in wet weight, due to extravascular fluid loss, of 0·3–2·8 g./g. tissue. In nine of the other experiments the figures for the gassed lungs were 0·3–3·5 g., increases which were 1·5 to 20 times those found in corresponding controls. In three experiments only were there no significant differences between the gassed and control lungs. In these three experiments any effects which might have been produced by gassing were overshadowed by gross pathological changes associated with massive haemorrhage into the alveoli and around the arteries and bronchi. This had occurred apparently as the result of obstruction to the circulation since fine fibrin clots were found to be present in various parts of the perfusion apparatus when this was subsequently examined.

In this connexion it may be noted that in our experience there was always haemorrhage of this kind, though not so massive, in all experiments in which there was spontaneous oedema, and it is possible that obstruction to the circulation was a factor contributing to uncontrolled oedema in all cases. In such experiments histological examination showed that the changes found in the control lungs were present in the test lungs as well. The differences were that in the gassed lungs oedema was generalized, while in the control lungs it had a patchy distribution and was often confined to one lobe or part of a lobe; and that peri-bronchial haemorrhage when present was more severe in the gassed lungs. Bronchial desquamation (described also by Daly *et al.*) was also present in gassed lungs which had been perfused for at least 2 hr. after gassing, making its appearance first in the smallest bronchioles and affecting the larger airways later.

It thus appeared from these results that the inhalation of phosgene could cause in isolated perfused lung tissue damage which led to pulmonary oedema; but under the conditions of test obtaining most frequently this was superimposed on an existing damage which could by itself cause pulmonary oedema, although this had a more restricted distribution.

Potassium

A rise in the plasma K above the control values occurred as an early and constant response to the inhalation of phosgene. Typical responses to a large and to a relatively small dose are shown in Fig. 2.

The plasma K normally tends to fall during the first 2 hr. of perfusion (Hebb & Nimmo-Smith, 1946) owing to the transfer to the lungs of an excess which is initially present in the circulation. Part of this is derived from the tissues during bleeding; but the greater part represents a loss from the lungs themselves which occurs while the circulation is stopped during the preparations for perfusion. In some experiments gassing was carried out before all of this excess had disappeared and in these the actual increase in plasma K produced by phosgene was not large (see Fig. 3). Nevertheless, the effect was the same in respect of the differences between the gassed and control lung curves.

Apart from the immediate change in the plasma K level an approximate estimate of the effect produced could be obtained in another way. In Table 1 the amounts of K lost and gained by each lung per unit weight of tissue have been calculated for the whole perfusion period. We have already shown that if there were no experimental interference the values for the lungs should be equal. Thus the differences found can be ascribed to phosgene.

It is probable that the actual differences were in fact much larger than the values obtained by the calculations used to construct Table 1, since these were based on the loss and gain of K from the plasma only. Thus a similar calculation

based on the whole blood values obtained in Exp. C showed that the uptake of K by the control lung was 8·9 mg./100 g. tissue and the loss from the gassed lung 10·3 mg./100 g. lung tissue, giving a difference of 19·2 mg., approximately 90 % larger than the value shown in the table. Unfortunately, in other experiments, the whole blood K was only estimated occasionally for control purposes and so a similar calculation cannot be applied to them.

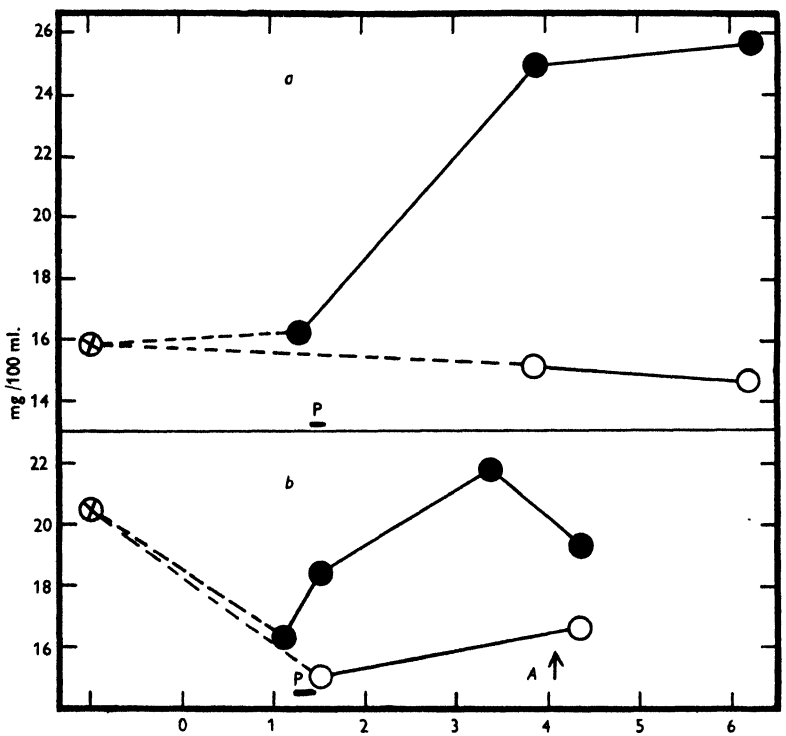

Fig. 2. Plasma K values in control (—○—○—) and gassed (—●—●—) lungs for two experiments. Ordinate: mg. K/100 ml. plasma. Abscissa: hours of perfusion. The initial values for each curve are for the unperfused blood which was collected 30 min. before perfusion started. *a*, 54 mg. phosgene into left lung 1 hr. 25 min. (P) after beginning perfusion; *b*, 13·2 mg. phosgene into left lung 1 hr. 14 min. (P) after beginning perfusion. At *A* injection of 10 μg. adrenaline into each lung circuit.

The rise in plasma K which typically occurred was not so rapid as that produced by adrenaline in experiments already described; nor did it subside so quickly. Usually after reaching its peak, the concentration remained at or near the same value for several hours, although in some experiments (such as that shown in Fig. 2*b*) it fell off gradually.

Control analyses showed, as we have already indicated, that the increase in plasma K was accompanied by a parallel increase in whole blood K. The K_t/K_0

ratio of the erythrocytes was usually of the order of 1·1 (in one experiment only it was 1·6), and it remained nearly constant except for a slight reduction in the presence of severe haemolysis. The error introduced by haemolysis in the

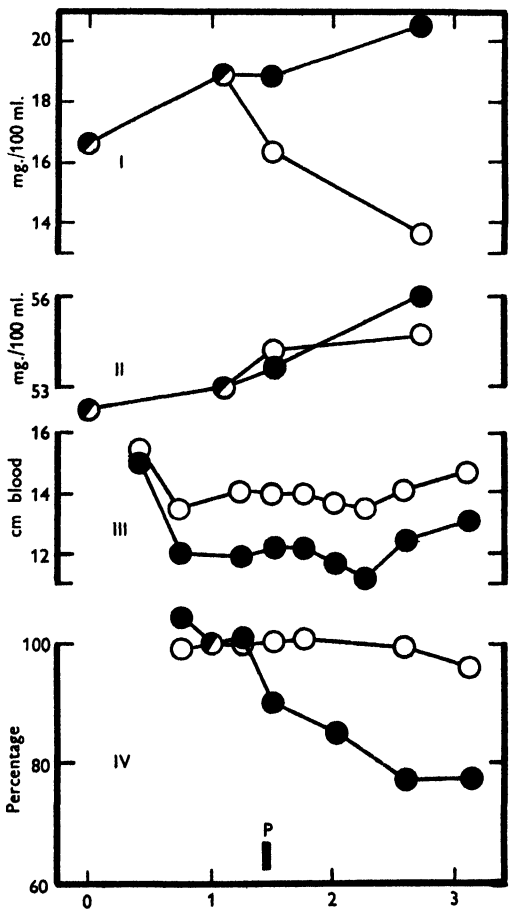

Fig. 3. Values for plasma K (I), blood haem Fe (II), pulmonary arterial pressure (III) and tidal air (IV) for control (—○—○—) and gassed (—●—●—) lungs of one experiment. Abscissa: hours of perfusion. 12·5 mg. phosgene (P) given 1 hr. 25 min. after beginning perfusion. Cross-circulation of the two lungs was maintained for the first 1 hr. 9 min. of perfusion; after that the circulations were separated as in other experiments.

The tidal air values are expressed as a percentage of the volumes obtaining for each lung at the beginning of perfusion.

estimation of the plasma K could, however, never have been more than a few per cent; nor could the exchanges of K between the erythrocytes and plasma have significantly affected the result except, as already indicated, to lead to an underestimate of the amounts exchanged between tissue and blood.

The rise in circulating K was not correlated with broncho- or pulmonary vasomotor responses to phosgene. Bronchoconstriction due to phosgene was of variable intensity and the reductions in tidal air observed ranged from 10 to 80%. On calculating the total K released into the plasma per 100 g. of lung tissue this value was also found to vary widely (between 4 and 35 mg.). This variation could not, however, be related to the variation in the intensity of the tidal air response. Similarly, it was independent of changes in blood pressure, since the release of large amounts of potassium into the circulation occurred in experiments in which no significant changes in pressure were produced as well as in those in which there had been a rise in pressure. There was, therefore, no evidence to suggest that the effect was due to ischaemia. This had been regarded as a possible cause since when the circulation is at a standstill the lungs do lose potassium (cf. Hebb & Nimmo-Smith (1946)).

From the evidence obtained it was most probable that the magnitude of the rise in plasma K depended upon the mass of tissue damaged by phosgene, since there was a rough proportionality between dose of phosgene and amount of K given up per unit of tissue (see Table 1).

TABLE 1. Gain and loss of K by control and phosgene-poisoned perfused lungs

Exp.	Perfusion time	Phosgene (dose in mg.)	Change in K (mg./100 g. tissue) Control	Gassed*	Difference
A	7 hr. 53 min.	44	+ 9·3	−33·9	43·2
B	6 hr. 10 min.	54	+ 3·9	−47·4	51·3
C	4 hr. 20 min.	12·5	+ 5·7	− 5·1	10·8
D	4 hr. 21 min.	13·2	+15·2	+ 6·5	8·7

+ = uptake by tissue; − = loss; values calculated from the plasma K concentration.
* Phosgene administered during second hour of perfusion in each experiment.

It might also be noted that the larger the increase in circulating potassium the longer was the rise maintained (compare Fig. 2*a*, *b*). From this, too, it appeared that the amount of K given up was an index of the quantity of tissue damaged, since evidence was found that tissue not directly exposed to the gas was able to take up some of the excess liberated. This was shown in several experiments, one of which is illustrated in Fig. 4, in which a cross-circulation was established between the lungs during gassing and for a short time thereafter. From Fig. 4 it may be seen that after separation of the two circuits a larger amount of the excess liberated by phosgene disappeared from the control than from the test system as perfusion proceeded. From this it would appear that the effect of the gas was exerted directly on the tissue and was not dependent upon humoral transmission.

The rise in the plasma K was an early effect which had generally reached a maximum before there was any sign of the pulmonary oedema to be observed 1–3 hr. after gassing. If it was due to tissue damage of a non-specific character

it might be expected that a similar rise in plasma K would also occur as a preliminary to oedema in the control lungs. This was not found to be the case,

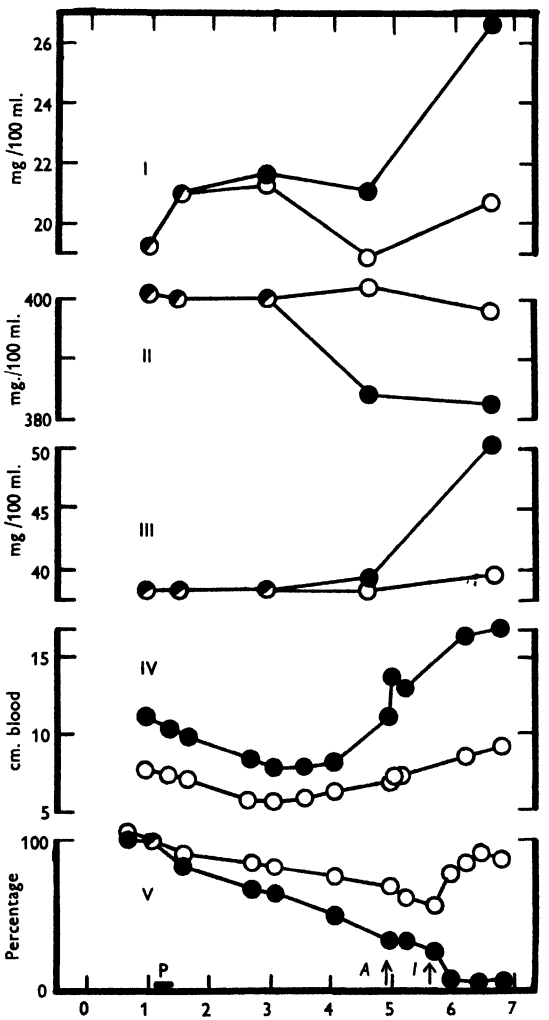

Fig. 4. Values for plasma K (I), plasma Cl (II), blood haem Fe (III), pulmonary arterial pressure (IV) and tidal air (V—percentage of initial values) for two lungs which were cross-circulated before and during gassing. The circulations were separated again 1½ hr. after beginning perfusion. Phosgene (13·2 mg.) was administered at 1 hr. 2 min. Control lung, —○—○—; test lung, —●—●—. At *A* injection of 1 μg. adrenaline into both circulations. At *I* inflation of each lung by positive pressure.

however, and the experiment of Fig. 2*a* may be cited as an example. In this experiment oedema developed in the control lung as a terminal event, and the wet weight increase at the end of the experiment was found to be 0·29 g./g.

but the K remained at low value. In the test lung where a similar change began much earlier the corresponding figure was 3·6 g./g. Analogous results were obtained in other experiments as well. The terminal increases in plasma K of Figs. 2*b* and 4 were due to injection of adrenaline and/or lung inflation (Hebb & Nimmo-Smith, 1946).

The fact that spontaneous oedema was not signalled by a rise in plasma K may not specifically differentiate it from phosgene-induced oedema. It was suggested earlier that stoppage of circulation in parts of the lungs might be a contributing factor to uncontrolled oedema; and if so a localized loss of potassium from the ischaemic tissue should follow. Such an effect might then be marked by the acceptance of excess potassium by the relatively larger bulk of tissue with a normal circulation. This suggestion cannot be regarded as more than speculative, since there is no certainty that spontaneous oedema originated in this way. Nevertheless, in one or two experiments in which there was sufficiently serious interference to the circulation to produce massive haemorrhage, the potassium in the blood remained at the high levels initially obtaining in lungs perfused in this way. In view of these considerations it may then be that the measurable rise in blood potassium and the more generalized distribution of alveolar exudate give a common indication that phosgene poisoning resulted in damaging a large proportion of the tissue, and that the same effect could be produced if in spontaneous oedema the same proportion of tissue were involved.

Chloride and water

Except for quantitative differences the water and chloride changes associated with the onset and development of pulmonary oedema were similar for both gassed and control lungs. The description which follows therefore refers to oedema under both conditions.

Changes in circulating Cl and water of the perfused dog lungs were carefully studied by Daly *et al.* (1946), who found that the combined loss of water and Cl from the blood was a certain indication of pulmonary oedema. In their experiments the event was preceded by a loss of water alone as shown by an initial increase in the concentration of both Cl and haemoglobin. It seemed probable that such water losses occurred as the result of evaporation, and this supposition was confirmed in those of our experiments in which we were successful in preventing such losses.

In these experiments there was no initial loss of water; the migration of water out of the blood was always accompanied by Cl; and the combined uptake of water and Cl by the tissue was indicative of an oedematous change which was progressive and gained momentum as it proceeded. Fig. 4 shows the change in haemoglobin and Cl in an experiment in which there were no measurable evaporation losses. In the experiment of Fig. 3, on the other hand, water losses due to this cause were equivalent to 2·5 % of the blood volume in each system.

Another finding of the earlier investigation was that with the development of pulmonary oedema the ratio of Cl to protein gradually fell so that Cl loss apparently preceded that of protein. After perfusion periods of 5–7½ hr. the ratio was reduced by 5–34% (outside limits). It was suggested that this was the result of a progressive increase in capillary permeability. For this view there was the further evidence that oedema fluid collected from the lungs at the end of an experiment had a Cl content which was higher and a protein content which was lower than the corresponding values in the terminal samples of plasma.

From a further examination of the changes in blood and plasma Cl there is evidence to indicate that there was another explanation for these results, namely that the fall in the ratio of Cl to protein was due to contamination of the plasma samples by haemolysis. The evidence was as follows.

In experiments in which there had been no losses of water by evaporation it was observed that coincident with the onset of haemoconcentration the plasma Cl, as well as the whole blood Cl, fell. The fall in whole blood Cl was to be expected since the cell concentration of the ion was usually less than one-half its concentration in the plasma, and the diminution of the volume of plasma relative to the cell volume would therefore have had that effect. On the other hand, the fall in plasma Cl was both unexpected and improbable since it indicated that Cl was entering the tissues at a concentration higher than that existing in the plasma. It had been observed, however, that there was a considerable degree of haemolysis in the centrifuged blood samples taken during the onset and progress of pulmonary oedema; this would by itself account for a reduction of the plasma Cl values owing to the lower concentration of Cl in the erythrocytes. Our suspicion that this was the cause of the fall in plasma Cl was borne out by evidence obtained in two experiments in which we estimated the amount of haemoglobin present in some of the plasma samples used for analysis.

In the experiment shown in Fig. 4 plasma obtained by centrifugation of the terminal blood samples was found to contain haem Fe equivalent in the case of the test lung to 7·3 ml. corpuscles/100 ml. plasma and for the control lung 1·7 ml. corpuscles/100 ml. plasma. Before the onset of haemoconcentration the whole blood Cl was 330 mg., plasma Cl was 400 mg./100 ml., and the red cell volume 33% .The cell Cl was thus 188 mg./100 ml. packed corpuscles. From these values it could be calculated that with the amount of haemolysis which occurred the dilution of the plasma in respect of Cl would be such that the contaminated samples would contain about 383 mg. in the case of the gassed lung and 395 mg. in the case of the control. The corresponding values obtained by analysis were in fact 382 and 398 mg. respectively which was a good enough agreement to indicate that the fall in plasma Cl which had been observed could be wholly accounted for in this way.

Further evidence that the fall in plasma Cl was due to haemolysis was provided by the finding that the fall in whole blood Cl could be wholly accounted for as the result of haemoconcentration and was not as large as would be expected if in addition to an increase in the percentage red cell volume the concentration of Cl in the plasma had also been reduced. Thus in one experiment in which the initial plasma Cl was 402, the whole blood Cl 306 and (by calculation) the corpuscular Cl 179 mg./100 ml. there was an increase in percentage red cell volume from 43 to 52·8 in the terminal sample. If there had been no change in corpuscular and plasma concentrations of Cl in the interval between the two samples, then in the second one the whole blood Cl should have been 293·5 mg./100 ml. By analysis it was found to be 294 mg./100 ml. On the other hand, the plasma Cl in the second sample was found to be 370 mg./100 ml. and if this were used as a basis for calculation then the whole blood Cl should have been much lower, or it must be assumed that the cell chloride had increased from 179 to 225 mg./100 ml., a result which is extremely unlikely.

Haemolysis would not only have led to a fall in plasma Cl; it would also have caused an apparent decrease in the ratio of plasma Cl to protein, partly owing to the relatively high protein content of the corpuscles. In the experiment which has just been considered, if it were assumed that the protein of the cells was 30% and that of the plasma 6·5% (values taken from results of Daly *et al.* 1946) then the degree of haemolysis present in the terminal plasma sample taken from the gassed lung circuit would account for an increase in plasma protein of about 1·8%. Thus, while the ratio of Cl to protein would have been 400 : 6500 (0·062) in the unhaemolysed sample it would be 382 : 8300 (0·046) in the haemolysed sample. This would represent a diminution in the value of 25% which is nearly equivalent to the largest reduction observed in the earlier investigation.

The evidence which has been given in respect of the change in whole blood chloride, and its close correspondence to the change expected to occur on the basis of the increase in percentage red cell volume, indicated that the haemolysis observed was an *in vitro* and not an *in vivo* change, which probably occurred during the centrifugation of the blood. In agreement with this we found, in one experiment in which we were able to analyse three successive samples of oedema fluid, that the Cl concentration in the three samples was 397 ± 1 mg./100 ml. and that in the initial plasma sample it had also been 397 mg./100 ml.; but that after the onset of oedema, the plasma values were 382 and later 370 mg./100 ml. The explanation of this finding appeared to be once again that owing to haemolysis the terminal plasma values were lower than those which really obtained in the circulation. In the same experiment it was found that the values for K concentration of the plasma and oedema fluid over the same experimental period were in agreement within the limits of experimental error.

This was to be expected in view of the nearness of the corpuscular K_i/K_0 ratio to unity and that haemolysis could consequently not produce any large changes in the plasma concentration of this ion. From these observations we concluded that the lack of correspondence between the composition of the oedema fluid and coincident plasma samples, in respect of Cl and protein, which had been observed earlier, could be wholly accounted for as the result of haemolysis produced in the preparation of the plasma samples. Because haemolysis was always present to some degree in the terminal plasma samples obtained in the investigation made by Daly *et al.*, this seemed to invalidate the previous evidence that Cl loss preceded protein loss from the blood. In fact, if haemolysis reached proportions equivalent to that found in some of our experiments then the order of change in the ratio Cl to protein found earlier becomes insignificant, and it would appear that the losses of protein and Cl were initiated at the same time and proceeded at the same rate.

On our interpretation the true significance of the plasma Cl changes which were found to be associated with the onset of oedema was the indication which they gave of an increased fragility of the corpuscles. In our experience the consistent occurrence of significant haemolysis in the separated plasma samples was as good a sign as any of the onset of pulmonary oedema. It was equally true that when this sign was absent, oedema was not found on other criteria. We can offer no explanation of this phenomenon, and while various causes might be suggested we have at present no evidence on which to base our speculations.

DISCUSSION

The genesis of pulmonary oedema in the phosgene-poisoned lungs presents a problem of special interest. In view of the low pulmonary arterial pressures (6–12 cm. water) which normally obtain before the onset of oedema this cannot be thought to be circulatory in origin. Similarly, at such low pressures, the colloid osmotic pressure of the blood should effectively counter any tendency to oedema unless, through damage, the capillaries suddenly become relatively more permeable to protein. Thus the onset of oedema is probably associated with the escape of protein from the circulation, and it would be expected that protein, chloride and water would diffuse outwards at their existing plasma concentrations. The evidence we have discussed is compatible with this view and there is no longer any reason to assume that the loss of water and crystalloids must necessarily precede that of protein.

Besides the occurrence of oedema the only other evidence of an effect of phosgene on the capillaries is the increase in capillary blood volume of the gassed lungs. Thus Daly *et al.* (1946) found that at the termination of an experiment the amount of blood which could be expressed from the vessels by positive pressure inflation is larger for gassed than for control lungs. This effect, which we find can be demonstrated for any pair of lungs perfused for 1 hr. or longer

after one has been gassed, is probably the result not only of an increased capillary blood volume but possibly also of a tendency for other intrapulmonary vessels of gassed lungs to be compressed under positive pressure inflation. The hyperaemia and congestion can be seen from direct inspection, and the lung haemoglobin figures of Daly *et al.* (1946) suggest that more blood is retained by control than by gassed lungs after drainage by inflation. An increased tendency for the capillaries of gassed lungs to collapse during positive inflation cannot be invoked in explanation of this phenomenon, since it is fairly certain that both normal unperfused lungs and also lungs perfused at constant input pressure have their capillaries emptied by a few mm. pressure (Daly, 1949). It seems that an explanation may exist in the response of other intrapulmonary (and possibly also of extrapulmonary) vessels to positive pressure inflation.

We have not sufficient evidence to assess the significance of the loss in tissue K which precedes the onset of pulmonary oedema. It seems probable that it is released as a result of damage to tissue directly exposed to the gas; the largest bulk of this is alveolar. It might be suggested, therefore, that the rise in blood K which follows exposure to phosgene is an early signal of the change in this tissue which is later responsible for its increased permeability and hence for the occurrence of pulmonary oedema.

The observation described in our previous paper that the perfused lungs normally take up K during the first 1 or 2 hr. of perfusion is at variance with the results of Wood & Moe (1942), who found that over the same period the whole blood and serum K of the blood-perfused dog lungs remains constant. The difference may be, however, because Wood & Moe employed positive pressure ventilation in their experiments (personal communication from Wood), whereas we have used only negative pressure ventilation. This suggestion is made on the grounds of our previous experiments in which it was found that a single inflation of the lungs by positive pressure may temporarily reverse the fall in plasma K which otherwise occurs.

SUMMARY

1. In isolated blood-perfused lungs of dogs the inhalation of phosgene may initiate oedema or augment oedema arising spontaneously in the course of perfusion.

2. The onset of oedema, with a latent period of 1 or 2 hr. after poisoning, is preceded by a marked increase in blood and plasma K above the control values. The increase occurs slowly and is maintained for long periods. This change is not observed as a preliminary to 'spontaneous' oedema.

3. Evidence is given that in the genesis of pulmonary oedema, water and Cl leave the plasma together at the existing plasma concentration of Cl.

4. Increased fragility of the red blood corpuscles in the perfusing blood is associated with the onset and progress of pulmonary oedema from phosgene and other causes. Evidence is given that the resultant haemolysis occurring during the preparation of plasma samples may be large enough to produce errors in the estimation of plasma Cl and protein.

These experiments were part of a programme of research carried out in this laboratory on behalf of the Ministry of Supply to whom we are indebted for permission to publish the results. We would also like to thank Prof. I. de Burgh Daly and Dr P. Eggleton for their interest and encouragement, and for valuable advice in the course of investigation. Dr O. A. Trowell gave us welcome assistance in examining histological material, and we should like to thank him and Miss Jean Banister who assisted in some of the experiments.

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ENZYMIC FORMATION OF ORTHO-TYRAMINE (*o*-HYDROXYPHENYLETHYLAMINE)

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The enzymic decarboxylation of meta-tyrosine (*m*-hydroxyphenylalanine) has recently been studied; it was found that extracts of mammalian kidney and liver, as well as acetone-dried preparations of *Streptococcus faecalis* R will catalyse this reaction (Blaschko, Holton & Sloane Stanley, 1949; Sloane Stanley, 1949). The enzyme preparations were incubated with the amino-acid under anaerobic conditions, and the occurrence of the decarboxylation reaction was demonstrated by two methods: (*a*) by measuring the formation of carbon dioxide, and (*b*) by comparing the pressor action of the amine formed with that of the synthetic product. The amine formed is meta-tyramine (*m*-hydroxyphenylethylamine).

The corresponding ortho-hydroxy derivative, ortho-tyrosine (*o*-hydroxyphenylalanine), is not a substrate of the bacterial enzyme (Sloane Stanley, 1949), but carbon dioxide is formed when the amino-acid is incubated with mammalian extracts under conditions exactly corresponding to those in which meta-tyrosine is decarboxylated (Blaschko, 1949). This observation suggested that ortho-tyrosine was a substrate of the mammalian DOPA decarboxylase.

Ortho-tyramine has since become available to us, and it was therefore possible to complete the study of the decarboxylation reaction by demonstrating the formation of ortho-tyramine. This amine is known to be a pressor agent (Barger & Dale, 1910). We have therefore incubated mammalian extracts anaerobically with ortho-tyrosine, measured the carbon dioxide formed, and compared the pressor action of the incubated extracts with that of synthetic ortho-tyramine.

A few experiments are also reported in which the properties of ortho-tyramine have been compared with those of its isomers, tyramine and meta-tyramine.

MATERIALS AND METHODS

The specimen of DL-ortho-tyrosine used was given to us by Dr A. Neuberger; for the gift of ortho-tyramine hydrochloride we are grateful to Dr E. J. de Beer, of the Wellcome Research Laboratories, Tuckahoe, N.Y.

The enzyme preparation was an extract of guinea-pig kidneys. The kidneys were ground with sand; 1 c.c. of M/15-sodium phosphate buffer was added for each g. of tissue. The suspension was centrifuged for 5 min. and the supernatant fluid was used.

RESULTS

Assay of pressor activity in kidney extract incubated with ortho-tyrosine

In the main experiment six conical manometer flasks with two side arms were used. Each side arm held 0·6 c.c. of guinea-pig's kidney extract. In the first three flasks, which served as blanks, the main compartment contained 1·8 c.c. of M/15-sodium phosphate buffer of pH 7·4; in the other three flasks the main compartment contained 1·8 c.c. of a M/33-solution of DL-ortho-tyrosine, dissolved in the same phosphate buffer. The manometer flasks, filled with nitrogen, were incubated at 37·5°. At the beginning of the experiment the contents of the side arms were tipped into the main flasks. Formation of carbon dioxide began at once and continued, rapidly during the first 9 min. and then gradually slowing down, until the reaction came to a standstill after 390 min. By that time a total of 88 cu.mm. of carbon dioxide had been formed in the three blanks, and a total of 2007 cu.mm. CO_2 in the three flasks which had contained ortho-tyrosine. The amount of carbon dioxide formed in the decarboxylation reaction was therefore taken as $2007-88=1919$ cu.mm. CO_2. On the assumption that all the L-ortho-tyrosine added to the three flasks had been decarboxylated, the amount of carbon dioxide expected was 1833 cu.mm. In other words, 1·05 moles of carbon dioxide were formed for 1 mole of L-amino-acid added. This shows that the reaction had gone to completion.

A subsidiary experiment was carried out at the same time in which a small amount of ortho-tyrosine was incubated with the extract. At the end of the incubation the retention of carbon dioxide was determined by acidifying with sulphuric acid. All figures for carbon dioxide in the main experiment have been corrected for this retention.

After 405 min. the incubation was terminated; the contents of the three blanks were transferred to a centrifuge tube and each flask was washed with 0·5 c.c. of water; the washings were added to the pooled contents. The contents of the other three flasks were treated in exactly the same way. The centrifuge tubes were at once incubated in boiling water for 5 min. under stirring, cooled and centrifuged. The supernatant fluids were decanted and stored in the refrigerator at −12°. After 3 days, immediately before the assay, the solutions were once again centrifuged, as more insoluble material had precipitated.

The amount of ortho-tyramine expected to be formed from the L-amino-acid added is $\frac{3 \times 1{\cdot}8 \times 137}{2 \times 33}$ mg. (the molecular weight of ortho-tyramine is 137). This is 11·2 mg. of ortho-tyramine, and this amount was assumed to be present in a total volume of $3 \times 3{\cdot}5 = 10{\cdot}5$ c.c. This gives a concentration of 1·065 mg./c.c. expected in the test solution.

Some of the extracts used in preliminary experiments caused a slight fall of blood pressure in the spinal cat; in the main experiment, therefore, the synthetic ortho-tyramine used in the assay was dissolved in the supernatant fluid obtained from the blanks; the concentration of ortho-tyramine chosen was 1 mg./c.c.

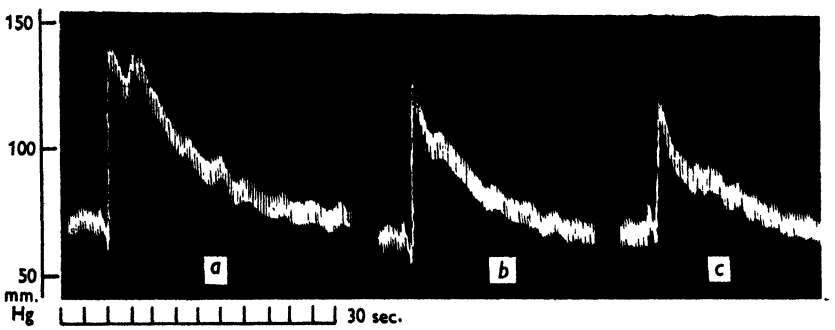

Fig. 1. Spinal cat; arterial blood pressure. Assay of ortho-tyramine formed by enzyme action. Intravenous injection at: (*a*) of 1·5 mg. ortho-tyramine dissolved in 1·5 c.c. of blank; (*b*) of 1·0 c.c. of test, calculated to contain 1·065 mg. ortho-tyramine; (*c*) of 0·8 mg. ortho-tyramine dissolved in 0·8 c.c. of blank.

The result of the assay is shown on Fig. 1. One c.c. of the extract incubated with ortho-tyrosine was injected at (*b*); this amount of extract was expected to contain 1·065 mg. of ortho-tyramine. The extract had a pressor action, and the blood-pressure rise was smaller than that after an injection of (*a*) 1·5 mg., and greater than that after an injection of (*c*) 0·8 mg., of synthetic ortho-tyramine.

The pressor action of ortho-tyramine

In agreement with Barger & Dale (1910), it was found that ortho-tyramine is much less active as a pressor agent than tyramine and meta-tyramine.

The action of ergotoxine. The effect of an injection of ergotoxine was to abolish the blood-pressure rise due to ortho-tyramine. Fig. 2 shows the responses to 10μg. adrenaline, to 5 mg. of meta-tyramine, and to 10 mg. of ortho-tyramine. In confirmation of earlier results (Blaschko *et al.* 1949) it was found that ergotoxine reversed the action of meta-tyramine on the arterial blood pressure; on the other hand, the effect of ortho-tyramine was abolished, but not reversed after ergotoxine.

The action of cocaine. Cocaine is known to have a potentiating effect on the pressor response to adrenaline; the response to tyramine, however, is depressed

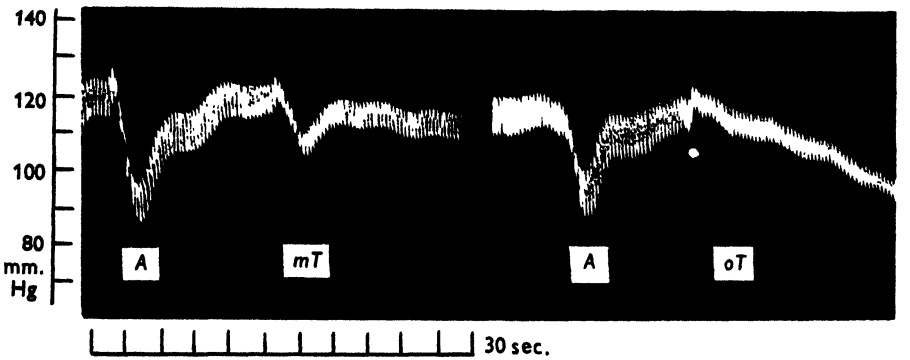

Fig. 2. Spinal cat; arterial blood pressure. The record was begun after the intravenous injection of 5 mg. of ergotoxine ethanesulphonate. Intravenous injections of: (*A*) 10 μg. adrenaline; (*mT*) 5 mg. meta-tyramine; (*oT*) 10 mg. ortho-tyramine.

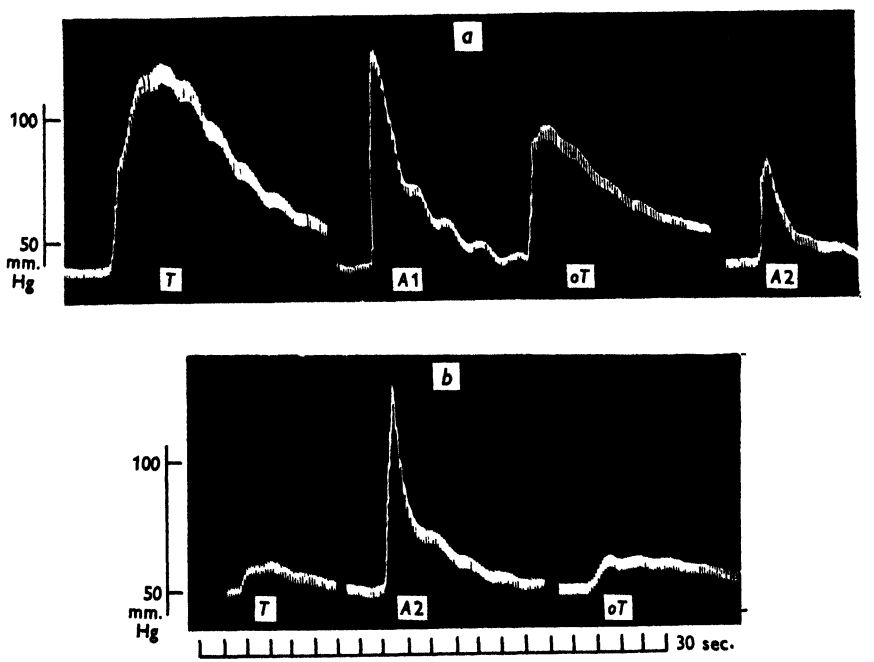

Fig. 3. Spinal cat; arterial blood pressure. (*a*) before, and (*b*) after, the intravenous injection of 10 mg. cocaine hydrochloride. Intravenous injections of 0·5 mg. tyramine (*T*), 10 μg. adrenaline (*A* 1), 5 μg. adrenaline (*A* 2) and 4 mg. ortho-tyramine (*oT*).

by cocaine (Tainter & Chang, 1926). It has been shown earlier (Blaschko *et al.* 1949) that the action of meta-tyramine is also depressed by cocaine, and the

experiment of Fig. 3 shows that the same holds true for ortho-tyramine: after an injection of 10 mg. of cocaine the pressor effect of ortho-tyramine was almost completely abolished.

DISCUSSION

The evidence reported above leaves no doubt that mammalian tissue extracts catalyse the reaction:

Ortho-tyrosine Ortho-tyramine

The quantitative data make it likely that the L-configuration of ortho-tyrosine is a substrate of the mammalian L-DOPA decarboxylase, as already suggested elsewhere (Blaschko, 1949).

It is interesting that the enzyme acts on the two isomers of tyrosine which have never been found in biological material, but that it does not act on tyrosine itself. The explanation is probably that normally the enzyme does not act on a monohydroxyphenylalanine, but on a dihydroxyphenylalanine. The possible significance of the enzyme in connexion with the synthesis of adrenaline and sympathin, as well as with the formation of homogentisic acid, has already been discussed elsewhere (Blaschko, 1942; Blaschko *et al.* 1949).

The low pressor activity of ortho-tyramine was already noted by Barger & Dale (1910); this finds its parallel in other series of sympathicomimetic compounds, e.g. in the series of monohydroxy-nor-ephedrines studied by Tainter (1932). In the series examined by Tainter, however, the meta-hydroxy derivative is much more potent than the para-hydroxy compound, and a similar difference has been found between the two Synephrines, where the meta-hydroxy compound (Neo-synephrine) is more potent as a pressor agent than the para-hydroxy isomer (Synephrine). In the tyramine series, there is no similar difference between the para- and the meta-hydroxy isomers.

In one point, meta-tyramine was found to differ from both tyramine and ortho-tyramine, namely, in the blood-pressure response after ergotoxine. The response to meta-tyramine was a blood-pressure fall, whereas the response to tyramine and to ortho-tyramine was abolished, but not reversed. Five phenylethylamine derivatives have been studied; of these three show the ergotoxine reversal: 3:4-dihydroxyphenylethylamine (Tainter, 1930), 2:5-dihydroxyphenylethylamine and meta-tyramine (Blaschko *et al.* 1949). These three compounds all contain the phenolic hydroxyl group in the meta position; the effects of the two amines without the meta-hydroxy group, tyramine and ortho-tyramine, were not reversed by ergotoxine. On the other hand, the pressor actions of Neo-synephrine and *m*-hydroxy-nor-ephedrine are not re-

versed by ergotoxine (Tainter & Seidenfeld, 1930; Tainter, 1932; Kuschinsky, 1930;Kuschinsky & Oberdisse, 1931), so that this property is not common to all substances containing meta-hydroxy groups.

SUMMARY

1. Extracts of guinea-pig kidney contain an enzyme which catalyses the enzymic formation of ortho-tyramine (*o*-hydroxyphenylethylamine) from L-ortho-tyrosine (L-*o*-hydroxyphenylalanine). The formation of carbon dioxide was measured manometrically; the pressor action of the amine formed was assayed on the spinal cat.

2. The pressor action of ortho-tyramine was abolished, but not reversed, by ergotoxine. After cocaine, the pressor action of ortho-tyramine was almost abolished.

This work was done during the tenure of a personal grant from the Medical Research Council by one of us (P. H.).

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THE *IN VIVO* FORMATION OF CITRATE INDUCED BY FLUOROACETATE AND ITS SIGNIFICANCE

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There is need in physiology for further convincing instances of the connexion of biochemical schemes based upon enzyme research with *in vivo* events in the living organism, in order to constitute the bridge between biochemistry and physiology. Up to the present, examples have been confined to the intermediary metabolism of pyruvate and vitamin B_1, to the effect of some narcotics, and to the enzyme cholinesterase. In this paper, we give an account of another important bridge, based upon the formation of citrate *in vivo* by injection of the poison fluoroacetate ($CH_2F.COO^-$). Since the C—F bond is very stable (Swarts, 1896) this compound cannot combine with SH groups as happens with the other halogen compounds bromo or iodo acetate.

According to a prominent biochemical scheme for the terminal stages of pyruvate utilization, as also of the oxidation of fatty acids, degradation occurs by synthesis from oxaloacetate and a 2-carbon active fragment of a 6-carbon tricarboxylic acid followed by subsequent degradation to a 5-carbon and then a 4-carbon dicarboxylic acid. In one turn of this cycle, known as the 'tricarboxylic acid' cycle (Krebs, 1943), oxidation of 1 molecule of pyruvate takes place.

On the basis of enzyme experiments *in vitro* on homogenates of guinea-pig kidney freed from residual substrates by centrifuging, Peters, 1948 and Liébecq & Peters, 1948, 1949 have advanced the working hypothesis that fluoroacetate can be activated in intermediary metabolism like acetate, and so built with oxaloacetate into a 6-carbon acid in the citric acid series; the foreign fluoro acid then 'jams' the cycle, leading to accumulation of citrate at this stage. The 'jamming' hypothesis explained the accumulation of citrate found by Liébecq & Peters in their kidney preparations in the presence of the substrates fumarate or fumarate + pyruvate, preparations which normally oxidize added citrate well. The hypothesis is consistent also with the earlier idea of Bartlett & Barron (1947) that fluoroacetate competed with acetate for an enzymic

* British Council Scholar.

component. It also provided an explanation of results by Saunders (1947) and colleagues who had drawn attention to the variation of toxicity of ω-fluoro-esters with a change in the length of the carbon chain; esters with even numbers

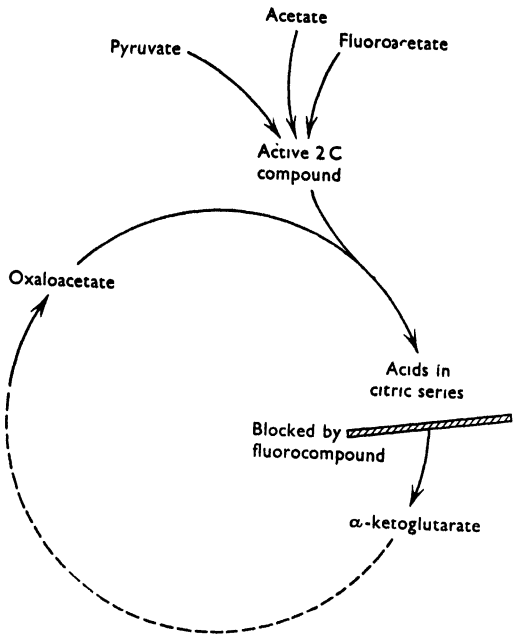

Fig. 1.

of C atoms were toxic and those with odd numbers were not toxic. This is a striking resemblance to the well-known scheme of Knoop, and in agreement with the modern ideas that fats are oxidized via the 'tricarboxylic' acid cycle. The hypothesis is present diagrammatically in Fig. 1.

METHODS

Animals. These were mostly albino rats of about 200 g. weight, which were fed upon rat cubes; some experiments were made with piebald rats.

Sodium fluoroacetate. (NaFlAc) was a pure sample kindly given by Dr B. C. Saunders (Cambridge), containing less than 0·1 % fluoride.

Administration of the poison. NaFlAc, 5 mg./kg., was dissolved in 0·5 ml. 0·9 % NaCl and injected intraperitoneally; the controls were similarly injected with 0·5 ml. 0·9 % NaCl. The animals were kept at room temperature and killed after 1 hr., unless otherwise stated, by decapitation.

Preparation of aconitase. In general, the method of Johnson (1939) has been followed both for the preparation of the enzyme from the pectoral muscle of the pigeon and from the heart of the rat. The tissue was quickly removed from the decapitated animal, cut into small pieces and ground in a mortar kept ice cold with sand and five parts of phosphate buffer pH 7·4 (0·1 M). When completely dispersed, the mixture was centrifuged for 8–10 min. at 4000 r.p.m. in the cold-room. The supernatant rose-tinted suspension was decanted and used as the 'stock enzyme'. Immediately

before use the 'stock' enzyme was diluted with a convenient volume of 0·1 M phosphate buffer in 0·9 % KCl or NaCl. The aconitase from rat heart kept at 2° C. rapidly lost activity; this loss is acentuated by dialysis.

Removal and treatment of the tissues. Whole brain was taken unless otherwise stated.

Heart	Freed from the residual blood, as far as possible.
Kidney	Both kidneys were used after removal of the capsule
Spleen	Freed from fat
Liver, *Lung*	Part of this was taken equivalent to 3–5 g.
Stomach, *Small intestine*, *Large intestine*	Freed from the contents, carefully washed with 0·9 % NaCl and blotted with filter-paper
Uterus	Freed from external fat
Blood	The total blood, collected from the animal into trichloracetic acid solution after decapitation.

Estimation of the citric acid. The method of Pucher, Sherman & Vickery (1936) was used with modifications, which are described below in full. It will be noted that the details differed from those given by Lardy (Umbreit, Burris & Stauffer (1945)). The tissues for estimation were quickly removed, weighed and ground in a mortar with 8 % trichloracetic acid (TCA); the volume of filtered extract was about 35 ml. TCA was used instead of metaphosphoric acid (Krebs & Eggleston 1943) because results with it were equally good and the extracts were clearer. For removal of 'pseudo' citrates the extract was boiled down with 3 ml. 50 % (v/v) H_2SO_4 to 17–15 ml. and then finally brought to 20 ml. by addition of water. The extracts could be stored at 1° C. without loss for at least 1 day. For the estimation, 0·2 ml. M-KBr and 1 drop of bromine water was added to 4 ml. of the TCA–H_2SO_4, boiled extract. At room temperature, 0·5 N-$KMnO_4$ was then added drop by drop with strong shaking in between each addition, until a reddish brown colour was reached; the optimum interval between drops was 60–90 sec. Usually 15–18 drops were sufficient; but sometimes, especially with blood and liver extracts, up to 60 drops were required. (This slow addition is very important if maximum yields of pentabromoacetone are wanted.) After transferring the tubes to the ice-bath and allowing to stand 5–10 min., the excess $KMnO_4$ was removed with H_2O_2 (6 vol.), which was added 1–2 drops at a time with very vigorous shaking and cooling in the ice-bath. As soon as the solution became colourless, diluted $KMnO_4$ (0·05 N) was added drop by drop to produce a persistent pale pink colour, which then turns yellow. Ice-cold water was then added to each tube to 10 ml., and then 13 ml. of purified petroleum ether, b.p. 65–70° C. The tubes were then shaken vigorously twice for 30 sec. with a 15 sec. interval, and returned to the ice-bath. Ten ml. of the petroleum-ether extract was transferred to a glass-stoppered cylinder, and kept in the ice-bath. Three ml. ice-cold fresh 4 % sodium sulphide solution were added, and the cylinders shaken twice for 30 sec. After standing in the ice-bath for 3–4 min., the petroleum ether was removed and the Na_2S extract was transferred to small centrifuge tubes. After short centrifuging (1 min. in an angle centrifuge) the Na_2S solution was transferred to a 1 cm. micro cell for reading. (We used a Hilger Spekker photometer and Ilford Filter 601.) Two standards containing Na-citrate equivalent to 196 μg. anhydrous citric acid were set up at the same time, which usually gave a reading of $0{\cdot}60 \pm 0{\cdot}02$ on the instrument. The values obtained for citrate were not altered by the 'alkaline heating' procedure of Breusch & Tulus (1947). The figures presented in the table are calculated for anhydrous citric acid and are the average of duplicate values agreeing within $\pm 0{\cdot}02$ (usually $\pm 0{\cdot}01$).

Estimation of α-ketoglutarate. The method of Friedemann & Haugen (1943) was followed; the final comparisons were made in the Beckman spectrophotometer.

RESULTS

A preliminary statement of these results has appeared elsewhere (Buffa & Peters, 1949).

In the main series of experiments, the organs were removed as quickly as possible after death without special cooling; this procedure is discussed below. Table 1 shows the results obtained, both as averages and as ranges over which

TABLE 1. Accumulation of citrate in the rat

	Citric acid (μg./g. wet tissue)					
	Controls			Poisoned		
Tissue	Animals tested	Average	Range	Animals tested	Average	Range
Blood	4	3	0–8	4	50	31–88
Brain	7	21	0–46	7	166	118–271
Heart	10	25	0–58	7	677	558–810
Kidney	8	14	0–48	8	1036	854–1220
Spleen	3	0	0	4	413	304–468
Liver	4	0·8	0–3	4	31	13–72
Lung	3	9	0–27	4	257	229–293
Stomach	3	37	19–53	4	386	297–457
Small intestine	3	36	25–53	4	368	157–535
Large intestine	3	21	10–38	4	248	193–338
Uterus*	3	217	179–276	4	207	158–300

* Virgin animals.

Dose NaFlAc, 5 mg./kg. body weight, dissolved in 0·5 ml. 0·9 % NaCl; controls injected with 0·5 ml. 0·9 % NaCl. Animals killed 60 min. after the injection.

the values fell. It will be noticed that there are relatively enormous accumulations of citrate in kidney, heart, brain and spleen. In blood and liver there is also an increase but this is quantitatively much smaller. Values in other organs are also interesting. The marked exception was in the virgin uterus, where little change took place. In skeletal muscle, it may be significant that in a comparison of the diaphragm and thigh muscle, the diaphragm which is not at rest showed the usual large increase (Table 2) but the thigh muscle showed little

TABLE 2. Distribution of citrate in the striated muscular tissue of the rat

Citric acid (μg./g. wet tissue)			
Diaphragm		Thigh	
Controls	Poisoned	Controls	Poisoned
0	400	5	41

Animals treated as those of Table 1; estimation on material from three animals.

change. There appears therefore to be a correlation with muscle activity. The increase in spleen is a puzzle which remains to be explained. Table 3 shows the distribution in the central nervous system for these animals; it will be seen that there is most in the cerebral hemispheres and least in the cerebellum.

TABLE 3. Distribution of citrate in the central nervous system of the rat poisoned with fluoroacetate

Citric acid (μg./g. wet tissue)		
Hemispheres	Cerebellum	Rest
123	39	89

Animals treated as those of Table 1; estimation on material from three rats.

Considering the changes as a whole the values for citric acid in the poisoned animals are so large (in one case seventy times) that the general conclusion that fluoroacetic poisoning causes large increase of citric acid seems clear enough. Nevertheless, it may be asked now whether these citric acid values represent the amounts in the tissue at death. In investigations of lactic acid content, it is well known that the greatest cooling precautions must be taken to get reasonably accurate results, because lactic acid is rapidly formed under anaerobic conditions (Davenport & Sacks, 1929). With citric acid, the evidence is that it is more easily formed under aerobic conditions (Coxon & Peters, 1949); hence it would not be expected to change rapidly upon death. A control experiment using two rats (Table 4) showed that there was no essential

TABLE 4. Rats poisoned with fluoroacetate; effect of the cooling of the organs with liquid air as compared with no liquid air treatment

	Citric acid (μg./g. wet tissue)	
Organ	No liquid air treatment	Liquid air treatment
Heart	677	523
Kidney	1036	874

Dose NaFlAc 5 mg./kg. body weight; killing after 60 min.

difference, if the tissues were removed rapidly and dropped into liquid air. This indicates that *in vitro* increases in citric acid formation are unlikely. In a final experiment six pairs of rats were taken and killed 2 hr. after injection of NaFlAc (10 mg./kg.); the tissues from one animal were dropped immediately into liquid air, and those from the other animal were removed 1 hr. after death

TABLE 5. Poisoned rat; effect of delayed extraction on the citrate content of the heart tissue

	Group I (6 rats) (immediate extraction)		Group II (6 rats) (delayed extraction)	
	Average	Range	Average	Range
Rat, wt. (g.)	235	220–248	236	224–248
Heart, wt. (g.)	0·888	0·662–1·013	0·945	0·683–1·172
Citric acid (μg./g. wet tissue)	898	762–1186	360	244–438

Male piebald rats; dose NaFlAc 10 mg./kg. body weight; killing after 120 min. Group I: heart rapidly removed and dropped into liquid air while still beating; after 2 min. extraction with trichloroacetic acid. Group II: heart removed from the carcass 60 min. after the decapitation, then same treatment as the others.

before dropping into liquid air; the dead animal was kept at room temperature during this hour. There was found to be a very large decrease post-mortem in citric acid content (Table 5).

Based upon the above information, it seems clear that these increases in citric acid *in vivo* are genuine, and that nothing was to be gained at this stage by elaborate precautions beyond reasonable speed in working.

Other animals. No extensive investigation has been made on other animals, but the results on a pair of pigeons, and a pair of guinea-pigs are given in Tables 6 and 7. They indicate that the rises in citric acid are not confined to the rat alone.

TABLE 6. Accumulation of citrate in the pigeon after poisoning with fluoroacetate

Tissue	Citric acid (μg./g. wet tissue)	
	Control	Poisoned
Blood	39	135
Brain	48	221
Heart	12	410
Kidney	36	1396
Liver	10	20
Gizzard	8	55
Breast muscle	34	82

Dose NaFlAc about 30 mg./kg body weight, dissolved in 1 ml. 0·9 % NaCl; control injected with 1 ml. 0·9 % NaCl. Animals killed after 60 min.

TABLE 7. Accumulation of citrate in the guinea-pig after poisoning with fluoroacetate

Tissue	Citric acid (μg./g. wet tissue)	
	Control	Poisoned
Blood	53	118
Brain	78	178
Heart	101	748
Kidney	36	1026
Spleen	85	313
Liver	9	13
Lung	82	242
Diaphragm	123	495
Thigh muscle	51	69

Dose NaFlAc 2 mg./kg. body weight, dissolved in 0·5 ml. 0·9 % NaCl, intraperitoneally; control injected with 0·5 ml. 0·9 % NaCl. Animals killed after 120 min.

Citrate concentration and time of death

It was thought that there might be some relation between the dose, time of death and citrate accumulation. A special experiment therefore was set up in which the dose of fluoroacetate was varied and the citrate accumulation noted at death; the results are given in Table 8. The time of death for 5 mg./kg. was unusual, as this is the 50 % lethal dose, and animals usually survive 12–18 hr. under our conditions. With this exception there was a tendency for lower survival time with the higher dose; but there was no simple relation for the amount of citrate. In the brain, the amount was approximately the same

throughout in this experiment, though in others values up to 350 μg./g. have been found. In the heart, the longer the survival the higher the value; in the kidney, the longer survivals were associated with less citrate.

TABLE 8. Citrate concentration and time of death in rats poisoned with fluoroacetate

(Albino rats of about 235 g.; all the fluoroacetate doses were dissolved in 0·5 ml. of 0·9 % NaCl; animals injected intraperitoneally and kept at room temperature (26° C. about); organs removed immediately after death.)

Dose NaFlAc (mg./kg.) ...	5	10	20	40	80
Survival time (hr., min.)	1, 37	28, 55	4, 22	1, 19	0, 48
Citric acid (μg./g. wet tissue):					
Heart	594	2175	1998	992	1052
Kidney	947	802	1920	311	316
Brain	214	191	247	226	221

In other experiments under the same conditions the following results were obtained:

Dose NaFlAa (mg./kg.)	10	20
Survival time (hr., min.)	3, 15	2, 10
Citric acid (μg./g. wet tissue):		
Heart	1122	1240
Kidney	850	1380
Brain	351	331

The uneven changes throughout the body in citrate formation, taken together with the *in vitro* evidence of its formation in the kidney homogenates, appears to eliminate the possibility that the citrate accumulations are produced by redistribution of citrate from the bones or elsewhere. The conclusion is drawn that the citrate is formed locally in the tissue cells, and that it does not diffuse out easily from these into the blood stream where the concentration is much below that in several of the tissues. It would be consistent with other indications that a tricarboxylic ion would permeate a cell wall even with greater difficulty than a dicarboxylic ion. As has been pointed out elsewhere (Liébecq & Peters, 1949), the failure to get citrate accumulation in brain tissue *in vitro* by treatment with fluoroacetate is explained by the lack of the activating acetate factor in the brain brei or homogenates. These do not utilize acetate. As there is increased citrate in brain *in vivo*, the acetate activating factor appears to be inactivated during the preparation of the enzymes. It has been reported (Bartlett & Barron, 1947) that guinea-pig brain tissue can oxidize acetate.

Reality of the tricarboxylic cycle. Though there is much evidence accumulating *in vitro* with enzyme preparations for the reality of the tricarboxylic cycle, it is surprising how little direct evidence exists. It is true that Orten & Smith (1937), confirmed by Krebs, Salvin & Johnson (1938) found that increases in urinary citrate followed feeding upon various acids of the 'cycle'; but we feel that the work reported here is more convincing proof of the utilization *in vivo* of this metabolic path. The increase in citrate takes place while the animal is still alive and, taken together with the evidence from kidney *in vitro*, seems a strong indication of the *in vivo* operation of the cycle.

The biochemical lesion responsible for the accumulation of citrate

The accumulation of citrate may be due to a block in the cycle either immediately at the stage of degradation of tricarboxylic acids or at some later stage in the 'cycle'. In the earlier war work in U.S.A. upon fluoroacetate, it was considered that its action had some relation to the tricarboxylic cycle; but this was not well-defined. Cori, Colowick, Berger & Slein (1945) found that aerobic formation of citrate from pyruvate and oxaloacetate was not inhibited by 0·05 M-NaFlAc, but they also found that citrate oxidation was less inhibited than phosphopyruvate oxidation, and in discussion considered 'that the exact point of action might be one of the steps in the dehydrogenation of pyruvate via the citric acid cycle'. Evidently it was the competitive behaviour with acetate which most impressed Bartlett & Barron (1947), and it was this hypothesis of action which they advanced.

At the time in which Liébecq & Peters (1948) first reported their experiments on washed homogenates of guinea-pig kidneys, and suggested the hypothesis that fluoroacetate could be built into the tricarboxylic cycle and so act as inhibitor of further oxidation, evidently other work on citrate was in progress. Thus Kalnitsky (1948) reported experiments with rabbit kidney which he interpreted to mean that fluoroacetate and barium salts increased the synthesis of citrate. Somewhat later Kalnitsky & Barron (1948) published an account of earlier work with rabbit kidney in which they showed citrate accumulation, though in our opinion the high residual values in their Table 2 make interpretation difficult, as in the case of some substrates it will not be justifiable to subtract these. All these experiments, including those with fluorobutyrate, can be interpreted on the hypothesis of Liébecq & Peters (1948) as supported by the results in this communication (see Fig. 1). Preliminary to the condensation with oxaloacetate there would be two possible points at which competition may take place: (1) at the enzyme which activates 'acetate' to the 'active 2-carbon stage', and (2) at the condensation of the 'active 2-carbon stage' with oxaloacetate. We suggest that the competition with some 2C compound from fluorobutyrate takes place at the latter point.

It should be pointed out that this activation stage is not yet clear as the nature of the 'active 2C' compound is not yet known; the possibility that phosphate takes an active part in the condensation reaction has been much ventilated. It is still doubtful whether the activation of acetate follows the same biochemical path in different tissues. Two points are difficult to reconcile with this; it is to be noted that the increases in citrate obtained *in vivo* in this work were less marked in liver than in other tissues, whereas Stern & Ochoa (1949), with a preparation from pigeon liver, recently got a direct condensation of acetate and oxaloacetate to citrate in presence of ATP and in virtual absence of aconitase.

How far do the present data indicate the actual enzymic block? Calculation shows that more citrate is accumulating in the animals than could possibly be synthesized as fluorocitrate or other fluoroacid from the amount of fluoroacetate administered. For instance, in a 200 g. rat dosed with 1 mg. (5 mg./kg.) $CH_2F.COONa$ and killed after 1 hr. as much as 7·2 mg. citric acid were found by analysis in the parts investigated (heart, kidneys, brain, spleen, stomach, small and large intestine). If we allow nothing for citrate accumulation in the rest of the body, then 10 μmol. fluoroacetate has given rise to 37 μmol. citrate. This proves that the effect is due to a 'jamming' of metabolism at some stage beyond that of citrate. Previously the possibility had not been excluded that the block might even occur as late in the cycle as the 4-carbon acid stage, i.e. at fluoro succinate (Liébecq & Peters, 1949); if this was so in fact, there should be also an accumulation at the intermediate 5-carbon stage (i.e. of α-ketoglutarate). Table 9 shows that no α-ketoglutarate was accumulating at a time when the concentration of citrate was high. Hence the block

TABLE 9. Failure of accumulation of α-ketoacids in the fluoroacetate-poisoned rat

	(μg./ wet tissue)		
	Citric acid	Na-pyruvate	α-Ketoglutaric acid
Blood			
Control	19·4	22·1	0
Poisoned	52·6	24·2	0
Heart			
Control	88·2	9·9	0
Poisoned	820	8·5	0

Female rat of 182 g. injected intraperitoneally with NaFlAc 5 mg./kg. dissolved in 0·5 ml. 0·9 % NaCl; female rat of 179 g. injected intraperitoneally with 0·5 ml. 0·9 % NaCl. The two animals were killed by decapitation after 70 min. and the blood and the heart tissue immediately extracted with 8 % trichloroacetic acid. 12 ml. of the TCA extract were used for the citric acid estimation and 8 ml. for the determination of the α-keto acids. The $E_{420m\mu}/E_{540m\mu}$ ratios were as follows: standard 48 μg. α-ketoglutaric acid = 1·82; standard 48 μg. Na-pyruvate = 1·42; blood, poisoned rat = 1·41; control rat = 1·39. This indicates that the α-keto acids in the blood were all pyruvic acid. The readings for the heart extracts were low, particularly at 420 mμ., and the ratios markedly on the pyruvate side.

must be at some point before oxalosuccinate, because this breaks down spontaneously to α-ketoglutarate. The problem therefore narrows down either to the stage isocitric-oxalosuccinate controlled by the isocitric dehydrogenase, or perhaps earlier at the stage of aconitase. This enzyme is held to catalyse the reactions citrate $\rightleftarrows$ *cis*-aconitate $\rightleftarrows$ *d*-isocitrate; at equilibrium approximately 80–89 % is considered to exist as citrate (Krebs & Eggleston, 1943; Johnson, 1939; Martius & Leonhardt, 1943), the later determinations approximate to 89 %. Previous work (Dixon & Needham, 1946; Bartlett & Barron, 1947; Liébecq & Peters, 1948, 1949) had indicated that no isolated enzyme was susceptible to fluoroacetate; but it seemed to be so important to be certain of

this, that the action of the poison upon isolated aconitase has been reinvestigated directly. Aconitase has been made from pigeon breast and rat-heart muscle by the method of Johnson (1939). Table 10 shows that there was virtually no

TABLE 10. Effect of NaFlAc on pigeon heart muscle aconitase *in vitro*

Substrate ...	*cis*-Aconitate				*cis*-Aconitate NaFlAc			*dl*-Isocitrate				*dl*-Isocitrate NaFlAc		
Time of incubation (min.) ...	0	20	60	120	20	60	120	0	20	60	120	20	60	120
Citric acid formed (%)	0	72·6	—	79·3	68	77·6	74·4	0	51·3	62·4	55·1	52·7	62·5	56·8

Aconitase stock solution 1:10, 3 ml.; sodium *cis*-aconitate 10 μmol. in 0·5 ml.; sodium *dl*-isocitrate* 20 μmol. in 0·5 ml.; NaFlAc, 1 mg. in 0·1 ml.; 0·9 % KCl to 4 ml.; pH 7·3, temperature 40° C., no shaking.

* Impure specimen.

difference between the amount of citrate formed from *cis*-aconitate or *dl*-isocitrate in presence or absence of fluoroacetate. The poison itself does not therefore change the equilibrium between the three substances. This confirms previous conclusions. There was still left the chance that *in vivo* the enzyme itself might have been poisoned. Accordingly, the action of the aconitase from a poisoned heart has been tried; and it has been found that it acts similarly to the normal (Table 11).

TABLE 11. Activity of the aconitase from the heart of the fluoroacetate-poisoned rat

Substrate ...	*cis*-Aconitate			Citrate		
Time of incubation (min.) ...	0	60	120	0	60	120
Citric acid (μmol.)	0	4 26	4·33	5·3	3·95	3·93
Citric acid (%)	0	85·2	86·6	106	79	78·6

Aconitase stock solution 1:10, 3 ml.; sodium *cis*-aconitate 5 μmol. in 0·5 ml.; sodium citrate 5 μmol. in 0·5 ml.; 0·9 % NaCl to 4 ml.; pH 7·3, temperature 40° C., no shaking. Citric acid content of the preparation 0·483 μmol.

It is concluded, therefore, that some compound, which is not fluoroacetate but which is formed from this, blocks the action *in vivo* either of aconitase or of isocitrate dehydrogenase; the most probable being the isocitric dehydrogenase. The most likely possibility is that the compound is a fluorocitric acid as this would be optically active; this is being investigated.

DISCUSSION

The relation of citrate accumulation to calcium

The biochemical lesion caused by the fluoroacetate is primarily the blocking of an enzyme which gives rise to increase in citrate. The fact that neither α-ketoglutarate nor other members of the 'cycle' (Cori *et al.*, 1945; Buffa, Liébecq & Peters, unpublished observations; Hutchens, Wagner, Podolsky & McMahon, 1949) will cure fluoroacetate poisoning suggest that death is not caused directly by the cutting off of the supply of energy by failure to syn-

thesize high energy phosphate as adenosinetriphosphate, because there are several steps in the 'cycle' not blocked which should be capable of this synthesis. Hence the question arises whether the citrate alone can be an adequate cause. So far as the blood is concerned there is not enough citrate to immobilize calcium and to cause tetany, and Foss (1948) did not find much change in the blood Ca of the goat during fluoroacetate poisoning. Consistent with this, in preliminary observations we have not found any increases in calcium accompanying the increases in citrate in rat's heart. So that the toxicity does not seem to be due to intracellular formation of Ca citrate. There is much older literature on the toxic effect of citrate itself (see Salant & van Hecht, 1915). Citrate is well-known to immobilize Ca ion physiologically, and the lowering of Ca ion concentration so produced initiates physiological activity (Adrian & Gelfan, 1933). There are attractive possibilities in this connexion in the explanation of the convulsive signs observed and of the changes in the electrical reactions in brain, Chenoweth & St John (1947); Brink, Bronk & Larrabee (1946), for instance, have reported that the immersion of frog (and squid) nerves in Na citrate solutions led to increases in excitability, which are also induced by a decreased concentration of Ca ion; the amounts of citrate employed (350 μg./ml.) are within the range of our findings. Hence the abnormal citrate concentration alone, in so far as it upsets the electrolyte balance by immobilizing Ca ion inside the cell, could be enough to account for the changed excitability preceding the convulsions. In a preliminary experiment (Table 12),

TABLE 12. Increase of citrate in the nervous system of the frog after injection of fluoroacetate

	Citric acid (μg./g. wet tissue)	
	Controls*	Poisoned†
Spinal nerves (brachial and sacral plexa)	152	232
Central nervous system	145	213
Heart	79	453

* Material from eighteen frogs, average weight 13·5 g., injected with 0·1 ml. 0·9 % NaCl in the abdominal cavity and killed after 15–18 hr. by freezing at −5° C. for 10–15 min.

† Material from eleven frogs, average weight 21 g., injected with NaFlAc 120 mg./kg. body weight in 0·1 ml. 0·9 % NaCl and killed after 12–14 hr. Heart taken for control of the poisoning.

using large numbers of frogs, it has been found that Na fluoroacetate in doses of 120 mg./kg.: (1) induced increases in citrate concentration in heart and brain; (2) killed 7/18 animals injected, in 10 hr.; and (3) gave a definite increase in citrate concentration in nerve. The increases found did not exceed 80 μg./g. tissue; but when it is remembered that the nerve sheaths were included in the analysis, it may be expected that the rise in the nerve itself is greater. It is highly significant that frogs surviving after 10 hr. showed toxic signs, viz. strychnine-like convulsions upon handling. The possible connexion of the tricarboxylic acid cycle, with normal nerve and muscle activity opens up

interesting relations of these cell enzymes with the inorganic ion investigations on nerve (Bronk, 1949).

From the pharmacological point of view, further substance is given to the suggestion (Peters, 1948) that in addition to 'les substances thioloprives', the term coined by Bacq (1946) for substances interfering with the normal action of SH substances, there should be considered to be a class of pharmacological substances which are 'cyclotoxic'. Whatever theory of the 'jamming' is finally proved, it seems sure that this formation of citrate *in vivo* is another useful bridge between biochemical enzymatic schemes and physiological events.

Note. While this paper was being completed for press our attention was drawn by Dr Ochoa to work by C. Martius (1949), *Liebigs Ann.* **561**, 227, in which independently using ox heart brei, he has reached a similar hypothesis to that of Liébecq & Peters (1948).

SUMMARY

1. The injection of Na fluoroacetate in doses of 5–10 mg./kg. into rats leads to large accumulations of citrate in most tissues within 1 hr.; these reached seventy times the normal value in kidney, the increases were large in heart, spleen, intestines, lung and brain, smaller in blood and liver and not present in the virgin uterus.

2. The diaphragm muscle showed a larger increase than the thigh muscle and the cerebrum than cerebellum.

3. In killed rats maintained at room temperature the citrate concentration in the heart diminishes.

4. Similar changes in citrate were noted in the pigeon and the guinea-pig.

5. There was no simple correlation between the amounts of citrate present in heart, kidney and brain, the dose and the time of survival; but there was a tendency for the amounts in heart to increase with increasing dose and those with kidney to diminish.

6. There was no α-ketoglutarate accompanying the increased citrate, nor were there any changes in pyruvate in either the blood or the heart.

7. Confirming other work, fluoroacetate has no action on isolated aconitase; the aconitase from poisoned heart also acts normally, hence it is not a direct action on this enzyme.

8. Of eighteen frogs injected with 120 mg./kg., seven died within 10–12 hr.; the remainder showed hyperactivity of the nervous system and accumulations of citrate in the heart, brain and spinal nerves.

9. These results show that fluoroacetate leads to a block in metabolism between citrate and α-ketoglutarate. They are regarded as support for the reality *in vivo* of the tricarboxylic acid cycle in metabolism, and for the hypothesis (of Liébecq & Peters), that a compound formed from fluoroacetate probably by way of the reactions of the cycle jams this at the citrate stage.

10. The significance of citrate is discussed in its relation to its influence upon the Ca ion and upon the hyperactivity of the nervous system.

We are indebted to R. W. Wakelin for technical advice, and to V. Bustin (Miss) for technical assistance; we are also grateful to Dr R. V. Coxon for help with estimation of α-ketoglutarate.

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PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

1–2 *July* 1949

An electrical drop recorder for students' use. By L. BERNSTEIN and J. C. BETTS. *Physiology Department, London Hospital Medical College, London*

The apparatus will record the passage across its associated electrodes of drops of liquid having a conductivity greater than 5 μmhos per drop, i.e. having a resistance less than 200,000 ohms. It operates from a.c. or d.c. supply mains of 220–240 V.

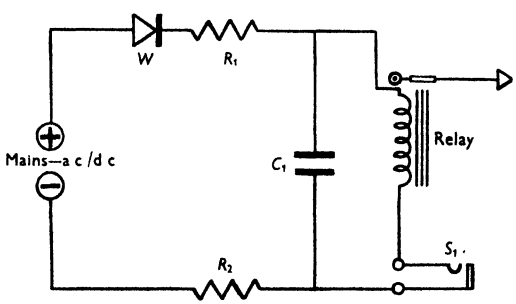

Fig. 1. *W*, Selenium rectifier, 250 V. 20 mA., half-wave. R_1, R_2, 47,000 ohms, carbon, $\frac{1}{4}$ W. $\pm 20\%$. C_1, 0·25 μF., paper tubular, 350 V. Wkg. Relay, G.P.O., type H.101.1.MT., 20,000-ohm coil.

The condenser C_1 is charged through the rectifier, *W*, and the resistances, R_1 and R_2, to the supply voltage. The passage of a drop of liquid across the electrodes discharges the condenser through the relay, which then closes. A writing point is attached to the relay arm. The current passing through the drop is about 5 mA.

The resistances R_1 and R_2 prevent any shock being obtained from the electrodes. These are plugged into the socket S_1 for use.

The recorder fits into a box, size $2\frac{3}{4} \times 2\frac{3}{4} \times 4\frac{1}{2}$ in., and the electrode system is described elsewhere in these proceedings.

The device is of low sensitivity and only provides a small amount of power. If it is desired to operate any accessory, such as a counter, this device must be operated from the supply voltage through contacts on the relay. It is not possible to operate accessory devices in series with the relay.

An electronic drop recorder and counter for low conductivity fluids.

By L. BERNSTEIN and J. C. BETTS. *Physiology Department, London Hospital Medical College, London*

The instrument is designed to record on a smoked drum the passage of drops of liquid across its associated electrodes. It can also be used to count the number of drops passing in any given period. The recorder is self-contained and can operate from a.c. or d.c. mains of 220–240 V.

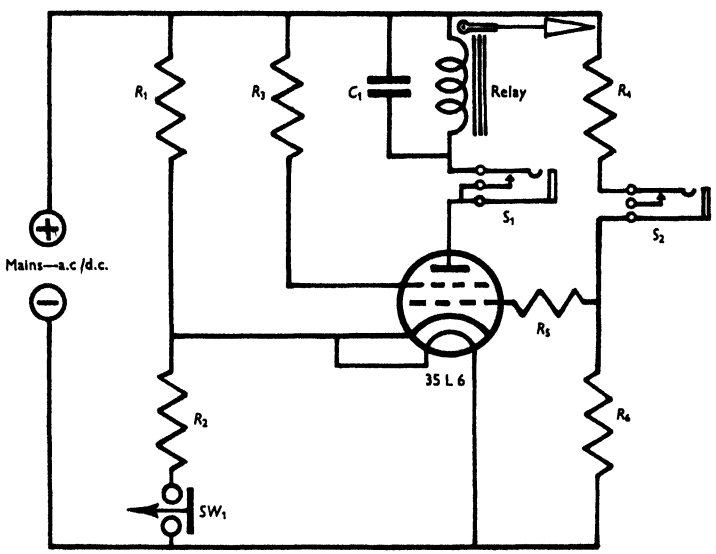

Fig. 1. R_1, 1400 ohms, line cord, 200 mA.; R_2, 500 ohms, carbon, 2 W., ±20%; R_3, 5000 ohms, carbon, 1 W., ±20%; R_4, 270,000 ohms, carbon, ¼ W., ±20%; R_5, 500,000 ohms, carbon, ¼ W., ±20%; R_6, 68,000 ohms, carbon, ¼ W., ±10%; C_1, 10 μF., 50 V. Wkg., dry electrolytic, tubular. Relay, G.P.O., type P27153, 400-ohm coil.

The anode current of the 35L6 valve is normally cut-off by applying its own heater voltage in suitable phase to the cathode, so providing grid-bias. The presence of a drop of liquid across the electrodes causes the grid voltage to swing towards the h.t. voltage and removes the bias on the grid. A current of about 70 mA. then passes through the relay, to the arm of which a writing lever is attached. The relay closes the switch, SW_1, placing R_2 across the valve heater. This prevents the anode current, which otherwise would flow through the heater, from damaging the valve. On a.c. supplies the valve conducts during positive half-cycles only, and the 10 μF. electrolytic condenser, C_1, prevents chattering of the relay.

The plate resistance of the valve is high, so that the insertion of other devices into the plate circuit is possible without affecting the operation of the relay.

A socket, S_1, is provided for the insertion of accessories, such as an electromagnetic counter which can be plugged in to count the drops.

The recorder is very sensitive, and will detect drops having a conductivity greater than $0{\cdot}2\,\mu$mhos, i.e. having a resistance up to 5 Megohms.

The electrode system is constructed of Perspex, the electrodes being of platinum wire. Details of construction are shown in fig. 2. Because the current passing through the drop is small—less than 0·6 mA.—base metal electrodes may be used. No shock can be obtained from the electrodes, as isolating resistors, R_4, R_5 and R_6, are provided in the recorder. The electrodes are plugged into the socket, S_2. The electronic portion of the apparatus fits into a small box, size $2\frac{3}{4} \times 2\frac{3}{4} \times 4\frac{1}{2}$ in. The electrode assembly is approximately $3 \times 1\frac{1}{2} \times 2\frac{3}{4}$ in. overall. The counter measures $2\frac{1}{2} \times 1\frac{1}{2} \times 1\frac{1}{2}$ in.

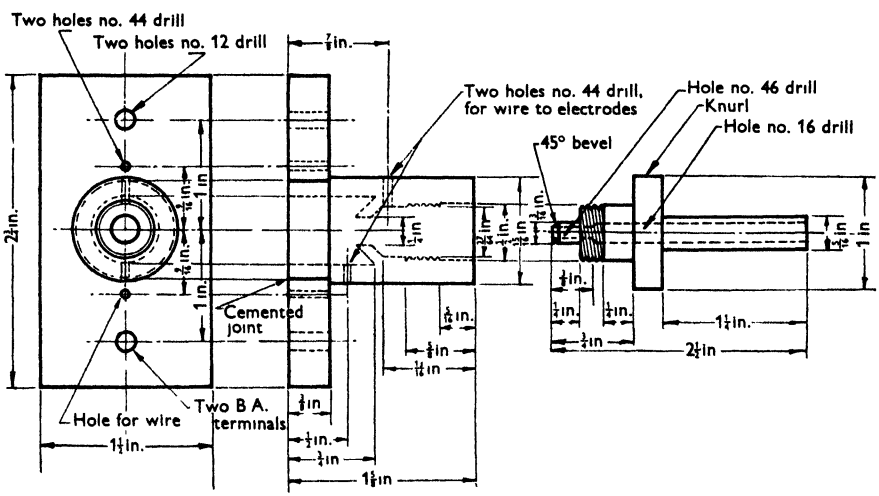

Fig. 2.

The apparatus has been found to have many applications. It is a very sensitive relay providing adequate power to operate warning or servo-motor systems. Examples of its uses are:

(1) Operating an alarm and a flow regulator when the fluid in a receiver reaches a predetermined level.

(2) Rotating a fresh receiver into position under the conditions of (1), or—by using a counting device such as a uniselector motor—when a predetermined number of drops has been collected.

(3) As a limit switch or counter for mechanical systems where the contacts must necessarily be very delicate, may have high contact resistance, and can only pass minimal current. The minimum operating current is about $40\,\mu$A.

Culture of lymphoid tissue *in vitro*. By O. A. Trowell.

M.R.C. Radiobiological Research Unit, A.E.R.E., Harwell

It is well known that when fragments of lymph gland or thymus are cultured *in vitro* by the usual techniques, of the lymphocytes which migrate out, some degenerate in a few hours and the majority die within 3 days.

With hanging-drop cultures of rat thymus or lymph gland fragments I have obtained best results in a medium of cock plasma and inactivated horse serum (equal parts) with addition of 200 mg.% glucose, on celloidin-coated cover-slips at 34° C. But even so, though a good proportion of the lymphocytes are healthy at 24–48 hr., the majority die within 4 days. Additions to the medium of embryo extract, thymus extract, bone-marrow extract, casein hydrolysate, xanthopterin, acetyl choline with eserine, were without benefit.

Bloom (1928) has claimed, in the rabbit, a much longer survival of lymphocytes if a fragment of embryo heart muscle is also growing in the medium, and (1937) the same result with a fragment of subcutaneous connective tissue. In the rat, I have been unable to confirm these findings.

In a new form of culture apparatus (devised by F. W. Randoll) the cultures were washed every hour with a large volume of oxygen-saturated serum, but the lymphocytes survived no better; so their death is not due to lack of oxygen, lack of serum foodstuffs or accumulation of metabolites.

In cultures made from suspensions of isolated thymus lymphocytes, survival is even worse, most of the cells dying in a few hours.

More success has attended the culture of whole lymph glands on the surface of a plasma clot, using a modification of the method introduced by Fell (1929) and used by Martinovitch (1938) for the culture of whole ovaries of young rats. For this purpose the lymph gland must not exceed 1 mm. diameter. The two sacral glands of a 2 weeks old rat are suitable. The gland is placed on the surface of a clot made from equal parts of cock plasma and rat serum, and is kept in an atmosphere of oxygen at 37° C. After an interval (usually 4 days) the gland is fixed and sectioned. Sections show the gland structure well preserved and the lymphocytes normal in appearance except for an occasional pyknosis. Mitoses are rarely seen; the gland does not grow but appears to survive in a healthy state. Glands have been cultured successfully for 7 days; longer periods have not yet been tried. Under these conditions of culture the initial pH of the medium was found to be 8·5–9·0. If 5% CO_2 is added to the gas phase the initial pH is about 7·5 but the cultures fail. A surprising degree of alkalinity appears, therefore, to be favourable.

The general conclusion is that, *in vitro*, lymphocytes survive in lymphoid tissue, but those which leave the parent tissue are doomed to die.

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Intracellular lymphocytes in thymus reticular cells and in fibroblasts cultured *in vitro*.

By O. A. Trowell. *M.R.C. Radiobiological Research Unit, A.E.R.E., Harwell*

Andrew (1945, 1946, 1947) has shown that most of the small lymphocytes which are ordinarily seen in the columnar epithelium of the small intestine are actually inside the epithelial cells and not, as hitherto assumed, between them. Andrew & Burns (1947) have shown that some of the lymphocytes in the tracheal epithelium are likewise intracellular. Darcy (1949) has found lymphocytes in the cytoplasm of the epithelial cells of rabbit submandibular gland homografts. The occurrence of intracellular lymphocytes in some other cells is therefore of interest.

In hanging-drop cultures of rat thymus very large thymus reticular cells (? epithelial) grow out in the form of sheets and also as isolated cells from the second day onwards, at which time many outwandered lymphocytes are still present. Some of these cells come to contain large numbers of lymphocytes in their cytoplasm. Most of these intracellular lymphocytes are in various stages of degeneration but a few appear to be normal. Popoff (1928) showed that these reticular cells which grow in thymus cultures do not store lithium carmine and concluded that they were not phagocytic. Murray (1947), however, thought that they were amoeboid and 'possibly phagocytic'. It is possible therefore that in this case the lymphocytes were simply taken up by phagocytosis.

In other experiments a culture of chick heart fibroblasts was grown alongside a thymus fragment, and after 2 days lymphocytes were seen within the cytoplasm of the outgrowing fibroblasts, again mostly in a degenerate condition. As chick fibroblasts are not ordinarily regarded as phagocytic the mechanism by which the lymphocytes entered these cells invites speculation.

It is characteristic of these intracellular lymphocytes in all situations that they are (in fixed preparations) surrounded by a clear unstainable halo.

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A class demonstration of the electrical and mechanical activity of single motor units in man. By G. GORDON. *Laboratory of Physiology, Oxford University*

The demonstration of rhythmic electrical activity in single motor units presents no serious problems, but their coincident mechanical activity is usually obscured by the contractions of other units in the same muscle. The vibratory subtetanic contraction of a single unit is usually only seen in minimal voluntary movements of human muscles (e.g. in the eyelids or in the fingers and toes).

The platysma, in a suitable human subject, has the advantage that its fasciculi spread out and separate widely in the lower part of the muscle, where they are attached to the skin and superficial fascia of the front of the chest. Slight voluntary contractions may give rhythmical movements of small isolated areas of skin in this region, and these can be shown to be due to the contractions of single motor units. The electrical activity of these units can be recorded by electrodes placed on the skin; but a much more satisfactory record, free of electrical spread from the intercostal muscles and the heart, can be obtained by inserting two fine needle electrodes in a fasciculus of platysma. The needles are insulated except at their extreme tips, and they are pushed through wheals of procaine to a depth of about 3 mm. They are separated by about 5 cm., and they are disposed along the line of a muscle fasciculus.

The action potentials are reproduced on a loud-speaker, and with each impulse the contraction of the unit imparts a visible movement to the needles.

The human platysma is variable in extent, but there should be no difficulty in finding a suitable subject for demonstration to a class of students.

Slow and rapid components in a flexor muscle. By G. GORDON and C. G. PHILLIPS. *Laboratory of Physiology, Oxford University*

Division of an extensor muscle group into a slowly contracting deep component and rapidly contracting superficial components is now well known (Denny-Brown, 1929), though this arrangement is not generally recognized to obtain among flexor muscles. It has nevertheless been found that the superficial motor units of the cat's tibialis anterior have a shorter contraction time than the deep and distal units of this muscle (Gordon & Holbourn, 1949). The part of the muscle in which slowly contracting units are found is redder in colour than the rest, and the fibres have a greater affinity for fat stains.

We have now compared isometric twitches of the slow part of tibialis anterior with those recorded from the whole muscle in the same preparation. This is made possible by the fact that the superficial quick part of the muscle can usually be separated to some extent from the deep slow part by dissection, without much damage to the slow part, and without interference with its nerve

supply. In the isolated slow part, under reflex drive, twitch responses of individual motor units can be clearly recognized; their crest tensions are of the order of 2·5 g. In quantitative observations on rate of contraction, an exact control of the temperature of the muscle was essential; the limb was enclosed in a thermostatically controlled moist chamber, and the observations were made when the temperature of the chamber and of the animal's body were the same. It is of interest that a fall of 10° C. in the temperature of the muscle may almost double the crest time of isometric twitches.

It was found that the slow part of the muscle, in preparations where its isolation is possible, gives simple twitches with a crest time of 25–34 msec. at 38° C. Twitches from the whole muscle show a sharp initial crest at 18–23 msec., with a hump during relaxation which corresponds to the crest time of the slow component. This is exactly comparable with the compound twitch recorded by Denny-Brown (1929) from the tendo achillis, which was made simple by removing either gastrocnemius or soleus.

Soleus, crureus and extensor digitorum longus, examined at the same temperature (38° C.), give crest times of 75, 58 and 19 msec. respectively. Our figures for soleus and extensor digitorum longus are considerably shorter than those given by Cooper & Eccles (1930); we attribute the difference to the higher temperature of the muscle in our experiments. In two experiments on each of these muscles, we have found that the crest times, at a fixed temperature, were constant to within 1·5%.

Preliminary observations on the reflex and cortical control of the two components of tibialis anterior suggest that the units of the slow part of the muscle have the lower central threshold, and may be engaged alone.

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An instrument for the stimulation of single hairs.

By R. Collin.
The Department of Human Anatomy, Oxford

In order to impart a standard movement to a single hair the instrument illustrated in Fig. 1 was constructed. The moving-coil magnet unit taken from a commercial loud-speaker is placed on a swivel mounting. The coil is resuspended and attached to a pin. In the end of this pin there is a fine hole through which a hair can be threaded.

Movement is caused by feeding in a sine-wave oscillation which can be varied both with regard to frequency and amplitude. The amplitude of the movement is measured by means of a micrometer screw gauge, and a calibration table

drawn up. The plane in which the hair is moved can be altered by turning the instrument on its swivel mounting.

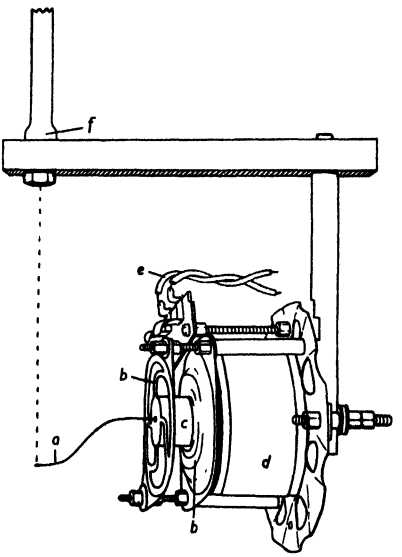

Fig. 1. *a*, pin with hole in end; *b*, suspension of moving coil; *c*, coil former; *d*, electromagnet; *e*, connexion to oscillator; *f*, swivel mounting.

The instrument has been used to stimulate single hairs both of rabbits and man.

Thanks are due to D. Taylor for his skill in assembling the unit.

Studies of the circulation through the liver. By P. M. Daniel and M. M. L. Prichard. *Nuffield Institute for Medical Research, Oxford*

The portal blood flow through the liver has been demonstrated in the living animal by means of serial angiographs, contrast medium being injected into a branch of the superior mesenteric vein. Rats, rabbits and cats have been used, and the circulation has been studied both in normal animals and also in animals subjected to various experimental procedures.

Amongst the vascular changes shown by the use of this technique have been:

(1) Constriction of the portal vascular tree in response to electrical stimulation of the hepatic plexus of nerves.

(2) The growth of the remaining portion of the vascular tree of the liver after partial hepatectomy.

(3) The extra-hepatic collateral vascular pathways which develop in rats with cirrhosis of the liver induced by carbon tetrachloride.

In addition, these studies include an investigation of the vascular and biliary systems of the liver in a number of animal species including man. Various injection methods have been used for this purpose.

The role of the neurohypophysis of the dog in determining urinary changes following the administration of sodium chloride. By W. J. O'Connor. *Department of Pharmacology, University of Cambridge*

Some properties of the cat's isolated cerebral cortex. By B. Delisle Burns. *National Institute for Medical Research, London*

In cats anaesthetized with chloralose, a layer of cerebral cortex containing grey matter with some of the underlying cortico-cortical fibres has been isolated from both the central nervous system and the adjacent cortex; the pial blood supply of this slab of tissue was the only connexion with the remainder of the animal. Usually, 3–4 cm.^{2} was isolated in the parietal region.

Such neurological isolation of the anaesthetized cortex caused the complete disappearance of all spontaneous electrical activity. Despite this, the isolated tissues were not dead; electrical stimulation excited responses similar to those obtained in the intact cortex. The isolated area would also respond to physiological excitation: if a narrow bridge of cortical tissue connected the otherwise isolated piece of cortex to the rest of the brain, spontaneous waves entered across the bridge and excited the isolated cells.

Local anaesthetics and the potassium ion. By O. Peczenik and G. B. West. *Department of Pharmacology, School of Pharmacy, University of London*

Large doses of procaine, amylocaine, cocaine, amethocaine and cinchocaine produce neuro-muscular block in the isolated rat phrenic nerve-diaphragm preparation at 20° C., and relative values are of the same order as those found for anaesthetic action in vivo. Doses of amylocaine and amethocaine producing a 50% inhibition are potentiated by 45 mg. of KCl, but doses of procaine are not potentiated. After all doses of procaine, KCl never prevents and often aids recovery of normal preparations, but produces block in diaphragms of adrenalectomized rats; after amethocaine and cinchocaine, KCl always prevents recovery and produces block. These results are in line with the actions of local anaesthetics on the inhibitory effect of acetylcholine on the rabbit's auricle (de Elio, 1948).

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The active principle of the adrenal medulla. By G. B. West. *Department of Pharmacology, School of Pharmacy, University of London*

Bülbring & Burn (1949) have shown recently that the discharge from the adrenal medulla is usually a mixture of noradrenaline and adrenaline. This observation has been confirmed by injecting the blood from the suprarenal vein

of a cat under chloralose (following stimulation of its splanchnic nerve) directly into the arteries supplying the denervated nictitating membrane and the denervated non-pregnant uterus of another cat under chloralose and cocaine. As continuous stimulation proceeds, the action on the uterus slowly disappears so that after 1 hr. up to 70% of the total active material secreted is noradrenaline. At this stage, an increase in the strength of stimulus temporarily produces more adrenaline.

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Bülbring, E. & Burn, J. H. (1949). *Nature, Lond.*, **163**, 363.

Para-aminohippurate excretion in the cat. By M. Grace Eggleton and Y. A. Habib. *Department of Physiology, University College, London*

It is generally accepted that a maximal rate of tubular secretion of para-aminohippurate (PAH) by the kidney occurs, both in man and dog, when the concentration in the plasma is above a certain level. This is seen also in the cat (creatinine clearance being used as a measure of glomerular filtration rate), the maximal rate being attained at about 10 mg. PAH/100 c.c. plasma. If the concentration is considerably increased, however, the amount secreted apparently diminishes again, until the PAH clearance may equal that of creatinine. Barclay, Cooke & de Muralt (1949) find that the human kidney behaves similarly with respect to diodone. In the cat the effect is reversible, the amount secreted rising again to a maximal value as the concentration in the plasma decreases. The depression at high concentration cannot, therefore, reasonably be regarded as a toxic effect; PAH is probably both secreted and reabsorbed, the latter process becoming dominant at high concentrations.

REFERENCE

Barclay, J. A., Cooke, W. T. & de Muralt, G. (1949). *J. Physiol.* **108**, 8*P*.

Some effects of decamethonium iodide on skeletal muscle. By Eleanore J. Zaimis*. *Department of Pharmacology, School of Pharmacy, University of London*

Decamethonium iodide, which is very potent in producing neuro-muscular block, has also a stimulating action on skeletal muscle. It produces contraction of the frog's rectus abdominis; in normal mammalian muscle its blocking action is preceded by a brief potentiation of the indirectly excited maximal twitch, and it can evoke a twitch-like response when suddenly injected close arterially (Paton & Zaimis, 1949).

Doses below the blocking level produce fibrillation of tibialis anticus and increase its response to single nerve volleys; in sensitive preparations the

muscle may go into tetanic spasm. These effects are potentiated by eserine and prostigmine in doses which alone are ineffective; these two drugs and dodecamethonium iodide, which are far more active than decamethonium iodide in inhibiting true cholinesterase, are less active in potentiating the twitch response of tibialis. The response of tibialis after denervation is a contracture similar to that elicited by acetylcholine, but more prolonged.

* With a grant from the Medical Research Council.

REFERENCE

Paton, W. D. M. & Zaimis, E. J. (1949). *J. Physiol.* **108**, 55 *P*.

Sympathin and the rat uterus. By Monica Mann. *Department of Pharmacology, School of Pharmacy, University of London*

It is well known that adrenaline exerts an inhibitory action on both pregnant and non-pregnant rat uteri. This response becomes excitatory in rats in full oestrus and in ovariectomized rats given oestrogen (when the vaginal smear contains cornified cells). Noradrenaline exerts a stimulating action in these states and also when the smear contains nucleated epithelial cells. Pre-sacral nerve stimulation always simulates the noradrenaline response.

A comparison of the actions of noradrenaline and adrenaline on the blood vessels of human skeletal muscle. By H. Barcroft and H. Konzett. *Sherrington School of Physiology, St Thomas's Hospital Medical School, London*

Allen, Barcroft & Edholm (1946) showed that infusions of adrenaline into the human femoral artery at 3 μg./min. caused a marked transient increase in the blood flow through the calf of the leg. Further analysis showed that this was due to a local transient dilator action on the blood vessels in the skeletal muscles.

Infusions of noradrenaline at 3 μg./min. cause decrease in calf-blood flow. Unlike adrenaline, noradrenaline has no transient vasodilator action in human skeletal muscle. The vasoconstriction is probably both in the skin and in the skeletal muscles.

REFERENCE

Allen, W. J., Barcroft, H. & Edholm, O. G. (1946). *J. Physiol.* **105**, 255.

Electromyographic study of patterns of activity in the anterior abdominal wall muscles in man. By W. F. FLOYD, *Department of Physiology*, and P. H. S. SILVER, *Department of Anatomy, Middlesex Hospital Medical School, London*

Muscle action potentials were recorded simultaneously from the muscles of the anterior abdominal wall in man. Surface electrodes were placed in pairs over the rectus, the external oblique and the internal oblique. (Near the inguinal canal, the internal oblique muscle is separated from the electrodes only by skin and the external oblique aponeurosis.)

The movement patterns of these three pairs of muscles were studied with the subjects recumbent and with the abdominal wall completely relaxed. When the head is raised off the couch, the rectus action predominates. When both legs are raised, all three muscles appear to contract equally.

In the erect posture, the internal oblique fibres are in a state of tonic contraction, which cannot be inhibited voluntarily.

In straining, which is a forced expiration against resistance, action of the obliques predominates: marked contraction of the rectus does not occur until a maximal effort is made.

The effect of isotonic potassium chloride solution on the electrocardiogram of the cat. By W. F. FLOYD and S. SALAMA. *Department of Physiology, Middlesex Hospital Medical School, London*

One of us (Floyd, 1947) has described the effect on the electrocardiogram (e.c.g.) of the frog of the application to the epicardium of pledgets of filter paper saturated with isotonic potassium chloride solution. According to the site of application, the resultant e.c.g. for this single ventricle heart resembles the dextro- or laevo-cardiogram (d.c.g. or l.c.g.) described by Hoff, Nahum & Kisch (1941) and Hoff, Nahum & Kaufman (1941) for the dog, cat and monkey.

We thought it worth while to repeat on the cat the experiments of Hoff *et al.* Our findings confirm their main observation that when isotonic potassium chloride solution is applied to the epicardium the resultant e.c.g. depends on the site and extent of the application. Records will be shown illustrating the effects of such applications to the anterior and posterior surfaces of both ventricles. The results are compatible with the hypothesis of Hoff *et al.* that the e.c.g. recorded by remote leads is due to the spread of activity over the epicardial surface.

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Focal potentials following antidromic excitation of spinal motoneurones. By THAIS H. BARAKAN, C. B. B. DOWNMAN and J. C. ECCLES. *Physiology Department, University of Otago, Dunedin, New Zealand*

In the spinal cord of nembutalized cats, focal potentials were recorded with a penetrating needle electrode after antidromic excitation of quadriceps motor nucleus.

In the white matter lateral to the nucleus the characteristic potential change was an initial positive wave followed by a large negative wave.

In the nucleus the negative wave was replaced by a large positive wave of similar time course. In this region all-or-nothing spike potentials of single units were recorded.

The late waves are associated with invasion of cell body by the impulse. Their lability may be due to changes in axon-soma junction block.

Responses of the colonic circulation in man to cooling the body. By J. GRAYSON. *Department of Physiology, The University of Bristol*

General body heating produces a vasoconstriction in the human bowel followed by a vasodilatation (Grayson, 1949). The same technique was used to investigate the effects of limb cooling.

When the arm was immersed in ice cold water, a variable and transient vasodilatation occurred at first always followed by a pronounced vasoconstriction and a fall in body temperature. With the circulation to the arm occluded, immersing the limb in ice cold water always produced a marked vasodilatation. On releasing the occluding pressure with the arm still in the water a rapid fall in body temperature and a pronounced vasoconstriction in the bowel occurred. Vasoconstriction was never observed in response to limb cooling whilst the circulation to the limb was occluded.

The vasodilator effect of cold on the bowel circulation is, therefore, not the result of direct cooling of the blood. The effect of circulating cooled blood appears, in fact, to be vasoconstrictor in the bowel.

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Grayson, J. (1949). *J. Physiol.* **108**, 47*P*.

Stimulating action of NH_4 ions on the perfused superior cervical ganglion of the cat. By JEAN BANISTER, CATHERINE HEBB and H. KONZETT. *From the Department of Physiology, Edinburgh University*

In experiments on the perfused superior cervical ganglion of the cat (method of Feldberg & Gaddum, 1934) it has been found that NH_4Cl has a stimulating

action on both normal and denervated ganglia. The ammonium salt is weaker than KCl in action and for equivalent effects (contraction of nictitating membrane) doses 2, 3 or even 6 times the dose of KCl may be required although sometimes as little as 0·6 mg. may give a good response. Contractions caused by NH_4Cl are slower in onset and longer in duration than those elicited by KCl. Like KCl, NH_4Cl is slightly potentiated by eserine. It may be that this is dependent upon the functional integrity of the pre-ganglionic endings since in denervated ganglia we have not been able to demonstrate such potentiation satisfactorily. The action of NH_4Cl on the sympathetic ganglion is probably analogous to its stimulating effect, described by Hermann *et al.* (1938), on the adrenal medulla.

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Circulatory changes in the spinal cord resulting from peripheral stimulation.

By E. J. Field, J. Grayson and A. F. Rogers. *From the Department of Anatomy and the Department of Physiology, The University of Bristol*

A fine copper-constantan thermocouple was introduced into the spinal cord of a rabbit at the level of the sciatic nerve to one or other side of the mid-line so that its point lay in or near the anterior horn of grey matter.

The heel on the side of the thermocouple was stimulated by nipping firmly over the calcaneum, adjusting the strength of the stimulus to obtain a slight withdrawal response. There was usually a rise in cord temperature unaccompanied by changes in body temperature. Occasionally a preliminary slight rise in cord temperature was followed by a marked and sustained fall. In three experiments heated thermocouples of the Gibbs type (1933) were employed. With the recording thermocouple at a temperature hotter than the body, stimulation usually produced a fall in cord temperature, indicating a local increase in blood flow.

Usually stimulation produces a vasodilatation and may be followed by a marked vaso-constriction.

REFERENCE

Gibbs, F. A. (1933). *Proc. Soc. exp. Biol., N.Y.*, **31**, 141.

The effect of pituitary tumours on renal function in man. By J. A. WATT and MARY PICKFORD. *Physiology Department, The University, Edinburgh*

Renal clearances of inulin and diodone were measured both before and after operation in patients suffering from chromophobe adenoma, and in acromegalics. The ability of the former to produce a water diuresis was also tested. Patients with chromophobe adenoma have very low clearances and excrete water poorly. Acromegalics may or may not have low clearances. Patients with chromophobe adenoma respond to water ingestion as do hypophysectomized dogs.

PROCEEDINGS

OF THE

PHYSIOLOGICAL SOCIETY

30 *July* 1949

Use of germanium rectifiers in condenser stimulators.

By I. C. WHITFIELD. *Physiology Department, University of Birmingham*

The problem of devising a simple student-apparatus for the production of single stimuli in nerve-muscle preparations where the pulse has to be repeatedly triggered, at short intervals, by a simple contact-switch, has been previously discussed (Whitfield, 1946). In the design of these stimulators it has been found that for stable operation under adverse insulation conditions it is essential that one side, both of the stimulating electrodes and of the switch, should be directly earthed. Also, if several such instruments are to be supplied from the same

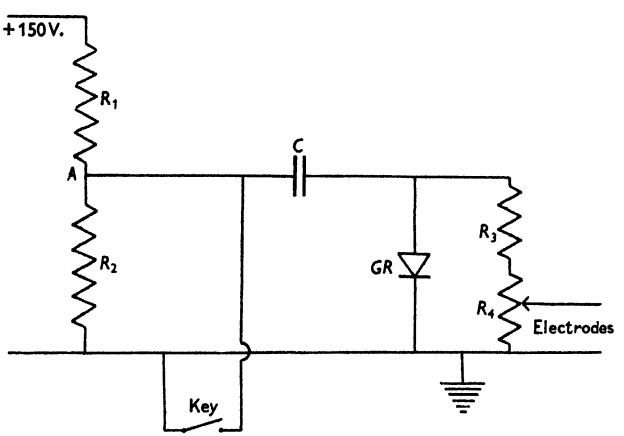

Fig. 1. $R_1 = 100{,}000$ ohms, $R_2 = 15{,}000$ ohms, $R_3 = 9000$ ohms, $R_4 = 1000$ ohms, $C = 0{\cdot}1\ \mu$F., GR = germanium rectifier type 3 GRK.

power source, this, too, should be at earth potential, since a floating power supply is liable to lead to interaction. A simple solution to this problem has been found in the high-inverse-voltage germanium crystal rectifiers which are now available. These rectifiers can be made to have an extremely low forward resistance while maintaining a back resistance of some thousands of ohms with an applied voltage of 20–50 V., the available variation of the constants being considerable in different types of rectifier. They combine these properties with

very small self-capacitance and physical dimensions. The type used in the apparatus described is the G.E.C. Type 3GRK which has been found satisfactory in class use over a period.

A suitable circuit, whose operation is described below, is shown in Fig. 1. With the key open there is a potential of about 20 V. across R_2, and the condenser is charged to this potential. On closing the key the condenser discharges through (R_3+R_4) in parallel with the back-resistance of the rectifier GR, this last being at least comparable in magnitude with R_3+R_4. The total voltage appears across R_3R_4, and a proportion of it can be tapped off across R_4. On releasing the key, the potential of point A rises and the condenser recharges, but, owing to the low forward resistance of GR, only a very small fraction of the voltage appears across it. In this way the ratio of 'peak make volts' to 'peak break volts' can easily be made as great as 60:1, and by suitable choice of R_1, R_2 even higher ratios may be obtained at the expense of a longer charging time. With a maximum output of 2 V. across R_4, which is more than sufficient for maximal stimulation under normal conditions, the 'break' stimulus remains well below threshold. The source voltage is not, of course, critical and may be varied, within the limits set by consideration of the factors mentioned above, by varying R_1 so that the voltage of the point A is kept the same. 150 V. has been chosen as a convenient value which enables the circuit to be combined in one instrument with a neon relaxation oscillator for repetitive stimulation, run from the same supply.

REFERENCE

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Lipid in the nephron of the cat.

By F. W. Gairns and S. D. Morrison.
Histology Division, Institute of Physiology, University of Glasgow

In a parallel histological and chemical investigation lipids were demonstrated in large amounts in the tubule cells of the nephrons of eighteen cats. Staining with Sharlach R and haemalum indicated that the fat concentration increased with the age of the cats. The location of the fat and the size of the intracellular fat globules varied widely with age. In three full-term foetal kidneys, Sharlach R produced only an orange coloration in the cortex; kittens from birth to 3 months showed definite fat globules, but the distribution was quite different from that of the adult. The lipid stained also with Sudan III, osmic acid and Nile blue and showed no birefringence. Excessive patency of the capillaries of the glomerular tuft was noted; the possible significance of this is being investigated.

Renal ether extractives as a percentage of kidney dry weight were significantly correlated with the dry weight of kidney, but the scatter was considerable.

The smooth muscle cell types and their associated elastic fibres in the female nipple. By F. W. GAIRNS and H. S. D. GARVEN. *Histology Division, Institute of Physiology, University of Glasgow*

In a previous study (Cathcart, Gairns & Garven, 1948) of the innervation of the female nipple, twenty-two cases were examined by the Bielschowsky-Gros method. The smooth muscle cell types and associated elements are now reported and illustrated.

Many of the smooth muscle cells are of the common spindle form; others are of a large, very branched type. This varies from spindle forms with small spurs, through Y shapes and star shapes, to very irregular forms, showing overlapping criss-crossing fibrils. Similar branched cells are present on the sinus-like veins.

The smooth muscle cells may lie in bundles or singly and may be surrounded by spirally arranged reticular fibres. Elastic fibres are closely associated with the smooth muscle cells. In some bundles up to 25% of the cross-sectional area may be elastic.

The functional significance of these branched, probably primitive, muscle cells requires further investigation.

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Cathcart, E. P., Gairns, F. W. & Garven, H. S. D. (1948). *Trans. R. Soc. Edinb.* **61**, 699.

Method of recording the movements of the horn and cervix of the uterus *in vivo*. By G. H. BELL, A. D. HITCHIN and BRENDA M. SCHOFIELD

Absorption of amino-acids from the small intestine. By J. B. WILSON

The role of the kidney in alloxan diabetes. By F. GRANDE, C. JIMENEZ-DIAZ and J. C. DE OYA

A comparison of adrenaline and noradrenaline on mammalian muscle. By G. B. WEST and ELEANORE J. ZAIMIS.* *Department of Pharmacology, School of Pharmacy, University of London*

The action of adrenaline in fatigued nerve-muscle preparations is thought to be primarily upon the muscle fibre itself, and the effects upon neuro-muscular transmission play only a small part in the enhancement of the twitch tension (Brown, Bülbring & Burns, 1948).

On the tibialis anterior of the cat, stimulated indirectly to maximal nerve volleys, both adrenaline and noradrenaline increase slightly the tension of normal muscle. Adrenaline also increases the tension developed by fatigued or partially curarized muscle, eliminates the post-tetanic potentiation when given intravenously before a tetanus, and produces potentiation of twitch tension when given with or after prostigmin; noradrenaline is much less efficient in producing these actions. Most of the effects possibly result from the liberation of potassium, adrenaline being more potent in effecting this release than is noradrenaline (D'Silva, 1949).

However, adrenaline and noradrenaline appear to be equally active in potentiating the stimulating and paralysing actions of decamethonium iodide.

* With a grant from the Medical Research Council.

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Sympathin and the cat uterus.

By G. B. West. *Department of Pharmacology, School of Pharmacy, University of London*

It is well known that adrenaline produces relaxation of the non-pregnant uterus and contraction of the pregnant uterus of the cat. Progesterone injections reverse from relaxation to contraction the response of the non-pregnant uterus to stimulation of the hypogastric nerve or to adrenaline injections.

In late pregnancy and in lactation, intra-arterial adrenaline and nerve stimulation produce relaxation, whilst intra-arterial noradrenaline is either excitor or produces a biphasic response (excitation followed by inhibition). Just before term, both amines are inhibitory *in vivo* and *in vitro*, and ratio values for equally active doses are similar to those obtained in non-pregnant animals. After dibenamine in pregnant cats, adrenaline and nerve stimulation produce the biphasic response when noradrenaline gives inhibition only. It is possible that the adrenergic mediator in this case is adrenaline or a mixture of adrenaline and noradrenaline (but not noradrenaline alone).

Lower requirement of gland extract for maintenance in sympathectomized-adrenalectomized than in adrenalectomized bitches.

By Mary F. Lockett. *Pharmacology Department, University College, London*

Three adrenalectomized and four sympathectomized-adrenalectomized bitches were treated with eucortone, received the same diet, and were kept under similar conditions.

Adrenalectomized bitches, in the first 3 months post-operative, required from 2 to 5 ml. eucortone daily. 0·5–1·0 ml. eucortone/day was found sufficient for the maintenance of sympathectomized-adrenalectomized bitches in the same post-operative period. The small differences between the weights and surface areas of these animals did not account for the large differences in their hormone needs.

In the later post-operative months, the doses of eucortone required to maintain the adrenalectomized-sympathectomized bitches in good health decreased progressively.

No suprarenal tissue was found post-mortem in the peritoneal cavities of two adrenalectomized-sympathectomized bitches, examined in the third and seventh post-operative months respectively. The post-mortem findings for the adrenalectomized animals were similar, but all were examined before the end of the fourth month after operation.

The effect of stimulation on the rate of exchange of potassium ions in the superior cervical ganglion of the cat. By N. Emmelin, F. C. MacIntosh and W. L. M. Perry. *National Institute for Medical Research, Hampstead, London*

Depolarization of cell membranes is known to be associated with increased transfer of potassium ions. We have investigated the rate of exchange of potassium in the superior cervical ganglion of the cat, using radio-potassium (K^{42}). The rate of uptake of potassium from the blood by the ganglion is significantly increased during preganglionic stimulation; the rate of output of potassium from a perfused ganglion previously loaded with radio-potassium is similarly affected. Antidromic stimulation through the post-ganglionic trunk did not produce any such increase in transfer of potassium. We have not, therefore, proved conclusively that the increased potassium exchange is due to excitation of the ganglion cells, since it might possibly be attributed to spread of the excitation through short post-ganglionic fibres to other effector cells in the mass of perfused tissue. We find this alternative explanation difficult to accept.

The time-course of events in the extraction of acetylcholine with trichloracetic acid. By W. L. M. Perry. *National Institute for Medical Research, Hampstead, London*

Chang & Gaddum (1933) recommended extracting acetylcholine with trichloracetic acid for 1–2 hr. Abdon & Hammarskjöld (1944) showed that release of acetylcholine from tissue by trichloracetic acid is 80% complete in 1 hr. I have

assumed their figures to be correct and the time-course of release to be exponential. Dale & Dudley (1931) showed that 60% of acetylcholine added to trichloracetic acid is destroyed in 19 hr. I have investigated the time-course of this reaction more completely and find that it also is exponential. At any moment during extraction this reaction naturally affects only that proportion of acetylcholine already released.

Mathematical combination of the equations of these two reactions shows that the maximum yield of acetylcholine is 90·6% and is obtained in 2·28 hr. The form of the combined curve is such that over 85% of the available acetylcholine is extracted in any period between $1\frac{1}{2}$ and $4\frac{1}{2}$ hr.

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The action of isopropylnoradrenaline on the cornu and cervix uteri.

By Brenda M. Schofield. *Department of Physiology, University College, Dundee*

Isopropylnoradrenaline (IPNA) inhibits the spontaneous contractions of the horn and cervix of the oestrous rabbit anaesthetized with nembutal, the effect being more prolonged on the cervix. The inhibition is quite marked with a dose of 0·2 mg. in a 2 kg. rabbit. Adrenaline given intravenously produces a contraction of the cervix in doses of 1 μg. upwards. The effect of this threshold dose is abolished by 0·2 mg. IPNA given at the same time. The cornual threshold for adrenaline, on the other hand, is 0·1 μg., but the contraction is not abolished by IPNA in doses up to 2 mg. Pitocin in doses of 0·02 unit intravenously produces a just perceptible contraction of both horn and cervix; this oxytoxic effect is abolished by giving 0·02 mg. IPNA. The effect of higher doses of pitocin is counteracted by proportionately higher doses of IPNA.

Sensory nerve impulses from the larynx.

By B. L. Andrew. *Physiology Department, University College, Dundee*

Nerve action potentials in the superior laryngeal nerve in the rat under urethane were recorded. With the trachea intact, bursts of afferent impulses occurred synchronously with respiration. When the larynx was isolated by tracheotomy, a stream of air blown up the stump of the trachea produced a continuous discharge of action potentials. This discharge was shown to contain two sensory components. First, large impulses arose from slowly adapting tension recorders

(probably muscle proprioceptors) mechanically linked to the vocal cords; these could be stimulated by movement of the cords whether produced by muscular or by external forces. Secondly, impulses of lower voltage originated from rapidly adapting superficial sense organs of the cords and pharynx. These were stimulated by touch and by movements of mucus produced by air currents.

An analysis of data relating to family size.

By MARGARET H. THOMAS.
Department of Physiology, University College, Dundee

Data obtained from a group of 198 medical students provided details of age and sex of members of their families as far back as the grandparents' generation. The average family of the grandparents' generation was 6·1, that of the parents 5·5, that of the present generation 2·7. All these differences are significant. In the present generation a family beginning with a boy and a girl (irrespective of order) tended to be limited to two, whereas if the sex of the first two members of the family was the same the family tended more frequently to be greater than two.

Studies in the circulation of the cornea in rabbits.

By F. C. RODGER

PROCEEDINGS

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24 *September* 1949

Perfusion of isolated tissues or organs. By J. A. HOLGATE. *Department of Physiology, School of Medicine, Leeds*

Many perfusion systems may contain two major sources of error, viz.:

(*a*) A lack of rigid control of perfusion pressure when variations occur in peripheral resistance due to alteration in vessel calibre.

(*b*) Absence of accurate information concerning the concentration reaching the perfused organ of the substance under investigation.

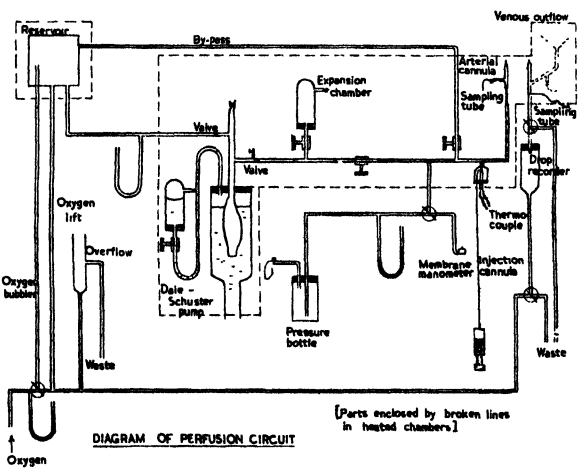

DIAGRAM OF PERFUSION CIRCUIT

The circuit below is an attempt to eliminate both of these.

(*a*) The pressure, both systolic and diastolic levels, is controlled by: (i) a double control of volume output of the Dale-Schuster pump by a stroke volume adjustment on the driving pump, and by a controlled pump expansion chamber; (ii) an accurately controlled elastic expansion chamber; (iii) a controlled by-pass enabling the total peripheral resistance to be maintained at a constant level.

(*b*) Injections in the flow have been made using an automatic ejector with a constant output rate. Samples of the injected substance mixed in the perfusion stream have been taken from the arterial cannula at various rates of

flow and the concentration estimated. From these results graphs concerning the degree of dilution with rate of flow have been constructed. Careful estimations of the quantity of injected material and the sum of the quantities removed in sampling and washing out have shown that 'pocketing' may occur in the dead spaces of a cannula system. Studies have also been made using dyes of various molecular weights to show the distribution of injections made into a moving stream in a cannula system as shown on the diagram, and these results have been correlated with those of concentration measurement and 'pocketing'.

Application of the infra-red analyser to respiratory studies. By A. HEMINGWAY

An apparatus for the automatic estimation of carbon dioxide in the presence of anaesthetics. By R. P. HARBORD, H. T. RINGROSE and S. T. ROWLING

The effects of carotid sinus reflexes on the renal haemodynamics in the dog. By R. A. KENNY and E. NEIL

Apparatus for recording motor activity in small animals. By P. HEY

Investigation of camera shake. By W. J. ALLEN

Vasodilatation in the forearm in response to heating the trunk. By K. E. COOPER and D. McK. KERSLAKE, Flight Lieutenants, R.A.F.

Radiant heat applied to the front of the trunk (Cooper & Kerslake, 1949) was found to cause an increase in forearm blood flow, which was apparent after 18 sec. of heating. In 1 min. of heating the blood flow increased by 1–2 c.c./100 c.c. forearm/min.

When the skin was blanched by electrophoresis of adrenaline (Barcroft, Bonnar & Edholm, 1947), there was no change in forearm blood flow during 1 min. of heating.

It is concluded that the vasodilator reflex previously described in the hand (Cooper & Kerslake, 1949) affects also the skin of the forearm.

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The effect of the haemodynamic state of the kidney on the excretion of secreted substances. By R. A. KENNEY. *Department of Physiology, School of Medicine, Leeds*

Urea and phosphate are known to be excreted by a 'three-component system' (Barclay, Cooke & Kenney, 1947*a*). In the case of both these substances it has been possible to demonstrate a correlation between the haemodynamic state of the kidney and the ratio between the clearance of urea or phosphate and the filtration rate. In the case of urea this relationship has been demonstrated in normal, hypertensive, nephritic, eclamptic and dehydrated human subjects (Barclay *et al.* 1947*b*; Kenney, Lawrence & Miller, in preparation; Kenney, 1949).

When the filtration fraction is high the ratio urea clearance: glomerular filtration rate is low and vice versa; the same holds good for phosphate (Barclay *et al.* 1947*a*). It is suggested that the haemodynamic change and the alteration in clearance ratio may both be manifestations of the preferential perfusion of an alternative renal circulation.

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The phenomenon of 'suck-back' in Douglas Bags. By H. R. NOLTIE. *Department of Physiology, School of Medicine, Leeds*

Judging by the literature no account seems to be taken of the suck-back which occurs when a tightly rolled up Douglas bag is opened again to the atmosphere. In bags used in this laboratory the volume sucked back ranges from $\frac{1}{2}$ to 6 l. Such a volume may or may not introduce an appreciable error, particularly where workers stipulate a time for collection of expired air rather than a minimum volume.

The author minimizes this dilution error by allowing the emptied bag to 'equilibrate' for a few seconds from a previously collected reservoir of expired air before closing the tap and stacking the bag for use. After sampling at the end of the experiment the contents are metered through a calibrated dry gas meter and then the suck-back measured on the meter and deducted. (The pointer may have to be blown away from the dead-beat point to allow full suck-back.)

Bacteriostatic action of heparin. By S. BRANDON STOKER. *Department of Physiology, Medical School, King's College, Newcastle-upon-Tyne*

In previous communications, Stoker & Pollard (1946*a*, *b*) reported that, as a result of trauma, heparin was released into the blood stream in measurable quantities.

The function of the heparin thus released has been investigated. It was found that in the presence of blood from the region of an injury or in the presence of pus, heparin had a marked bacteriostatic action. This action was not shown by the addition of heparin to pure strains of *Staph. aureus* but was exhibited when the culture was contaminated with a minute addition of pus or trauma blood. The inference is that a co-factor present in trauma blood or pus is necessary for heparin to act as a bacteriostatic agent. This action has been confirmed by the clinical use of heparin (0·44–0·05 unit per ml. in normal saline) in the local treatment of septic conditions. The pain, the congestion and oedema associated with inflammatory conditions and burns are relieved.

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The responses of denervated nictitating membrane to sympathomimetic amines. By MARY F. LOCKETT. *University College, London*

The responses of nictitating membranes have been examined, in spinal cats, 6–8 days after both preganglionic and postganglionic denervation.

Nictitating membranes, after preganglionic denervation, showed a lower threshold of sensitivity to L-adrenaline than did membranes after postganglionic denervation, in the corresponding animals. The reverse was true for threshold sensitivity to DL-noradrenaline; noradrenaline lowered the tone of preganglionic denervated membranes.

Increase in sensitivity to sympathomimetic amines appeared 4–6 hr. after preganglionic denervation. Six to eight days after operation, these membranes were more sensitive to β-phenylethylamine, tyramine, amphetamine and ephedrine than were the normal or the postganglionic denervated membranes of the corresponding animals.

The effects of postganglionic denervation of the nictitating membrane on the responses to noradrenaline and adrenaline (Bülbring & Burn, 1949) and to tyramine (Burn, 1932) are known. The changes produced by postganglionic denervation in the responses of nictitating membrane to β-phenylethylamine resembled those of tyramine; sensitivity to amphetamine was decreased.

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The use of Priscol (2-benzyl-imidazoline-hydrochloride) as a vasodilator in man. By W. J. ALLEN. *Physiology Department, School of Medicine, Leeds*

Measurements of the blood flow in the limbs (by means of plethysmographs) and of the skin temperature, supplemented by visual and other information, showed that intramuscular doses of Priscol of 40–80 mg. (particularly the larger dose) produced moderate or good vasodilatation in vasospastic conditions, whereas well-marked organic arterial diseases responded poorly, if at all.

Unexpectedly feeble dilatation occurred in the skin of many healthy limbs which were similarly tested.

In nearly all the experiments the effect of the drug on heart rate and blood pressure was small and unpredictable.

The effect of carotid sinus reflexes on cardiac output. By R. A. KENNEY, E. NEIL and A. SCHWEITZER. *Departments of Physiology, School of Medicine, Leeds, and University College, London*

Dogs used in these experiments were anaesthetized with nembutal (i.p. 40 mg./kg. body weight) or chloralose (i.v. 0·1 g./kg. body weight). Cardiac output was determined by the direct Fick method, O_2 usage being estimated by closed circuit spirometry or by the open method (Douglas, 1911). Arterial and right auricular samples were analysed for O_2 content, using the Van Slyke manometric apparatus.

Temporary occlusion of the common carotid arteries in intact or vagotomized dogs did not alter the cardiac output. This is contrary to the findings of Charlier & Philippot (1947).

Alteration of the intrasinusal pressure by means of a pump perfusion circuit did not cause any change of cardiac output in intact animals. After vagotomy, the initial results of raised intrasinusal pressure were more difficult to interpret because reflex apnoea, lasting sometimes for 20 sec., was wont to occur; when rhythmic breathing was resumed, however, the cardiac output was little changed, despite the maintenance of raised intrasinusal pressure.

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Evidence of the contribution of aortic chemoceptor mechanisms to the McDowall reflex. By J. C. G. COLERIDGE, R. A. KENNEY and E. NEIL. *Department of Physiology, School of Medicine, Leeds*

Section of the vagi in animals which have suffered a moderately severe haemorrhage may cause a fall in arterial blood pressure (McDowall, 1925). Comroe

(1939) has suggested that this fall of blood pressure is due to the interruption of a tonic discharge of impulses from the aortic chemoceptors.

This suggestion has been investigated in animals suffering from the effects of haemorrhage, by studying the effect of blocking the vagal impulses before and after selective elimination of the aortic chemoceptors by intraventricular injection of 0·5 N-acetic acid (Gernandt, 1946). The fall of arterial blood pressure occasioned by temporary interruption of vagal impulses is very much reduced or can no longer be elicited after eliminating the chemoceptors.

It is interesting to note that section of the carotid sinus nerves in cats, following haemorrhage, also causes a fall in arterial blood pressure, indicating that carotid chemoceptors additionally are involved in the maintenance of vasoconstrictor tonus in these conditions.

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The effects of intravenous infusion of mixtures of L-adrenaline and L-noradrenaline in man. By C. DELARGY, A. D. M. GREENFIELD, R. L. MCCORRY and R. F. WHELAN

Circulatory changes in muscle and skin to bladder distension in spinal men. By D. J. C. CUNNINGHAM, L. GUTTMANN, D. WHITTERIDGE and C. WYNDHAM

Structure of choline derivatives showing nicotine-like stimulating activity. By P. HEY

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